

Cyclins in Breast Cancer

Kirsimari Aaltonen

Department of Oncology
and
Department of Obstetrics and Gynecology
Helsinki University Central Hospital
University of Helsinki
Helsinki, Finland

ACADEMIC DISSERTATION

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Supervisors: Professor Carl Blomqvist, MD, PhD
Department of Oncology
Helsinki University Central Hospital
University of Helsinki

Docent Heli Nevanlinna, PhD
Department of Obstetrics and Gynecology
Helsinki University Central Hospital
University of Helsinki

Reviewers: Professor Seppo Pyrhönen, MD, PhD
Department of Oncology and Radiotherapy
Turku University Hospital
University of Turku

Professor Tapio Visakorpi, MD, PhD
Institute of Medical Technology
University of Tampere

Official opponent: Docent Minna Tanner, MD, M.Sc, PhD
Department of Oncology
Tampere University Hospital
University of Tampere

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ABSTRACT

Breast cancer is the most common malignancy in women in Western countries. It is a heterogeneous disease with varying biological characteristics and aggressiveness. Although understanding of breast cancer carcinogenesis is increasing and classification has improved, many aspects of tumorigenesis, molecular mechanisms, and prognostic evaluation remain to be elucidated. Family history is one of the strongest predisposing factors for breast cancer. In most populations, the known susceptibility genes explain only around 25% of all familial breast cancers. At least part of the unknown familial aggregation may be caused by several low-penetrance variants that occur commonly in the general population. Cyclins are cell cycle-regulating proteins that interact with cyclin-dependent kinases. Cyclin expression oscillates during the cell cycle and is under strict control. In cancer cells, cyclin expression often becomes deregulated, leading to uncontrolled cell division and proliferation, one of the hallmarks of cancer.

In this study, we aimed to investigate the role of cyclins in breast cancer predisposition, pathogenesis, and tumor behavior. The suitability of the tissue microarray (TMA) technique for cyclin assessment was studied in 200 breast cancer cases. Cyclin A immunohistochemistry was evaluated both on traditional large sections and on TMA, and the reproducibility of two readers' results was examined. The concordances of the findings from large sections and TMA and of the two readers' results were good. Histopathological correlations of cyclin A and correlations with prognosis were similar for both large sections and TMA. These findings indicate that TMA is a reliable method for studying cyclin expression in breast cancer.

The expression of critical cell cycle G1/S transition phase regulators cyclin D1 and E were studied among 1348 invasive breast cancers on TMA. Familial BRCA1/2-negative tumors had significantly more often low cyclin E and high cyclin D1 expression than BRCA1/2-related or sporadic tumors. In a logistic regression model, cyclin E and D1 expression, estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) status, and early onset of disease were the factors most clearly distinguishing BRCA1 patients from other familial breast cancer patients. Unique cyclin E and D1 expression patterns among familial non-BRCA1/2 breast cancers as compared with BRCA1/2-related or sporadic

tumors may reflect different predisposition and pathogenesis in these groups and help to differentiate mutation-positive from mutation-negative familial cancers.

When assessing cyclin expression with regard to breast cancer characteristics and prognosis, high cyclin E expression was associated with an aggressive breast cancer phenotype and was an independent marker of poor metastasis-free survival. High cyclin D1 was associated with high grade and high proliferation among ER-positive but with low grade and low proliferation among ER-negative breast cancers. Among ER-positive cancers not treated with chemotherapy, high cyclin D1 showed a trend towards shorter metastasis-free survival. These results suggest that different mechanisms may drive proliferation in ER-negative and -positive breast cancers and that cyclin D1 has a particularly important role in tumorigenesis of hormone receptor-positive breast cancer.

The association of a commonly occurring cyclin D1 gene (CCND1) polymorphism, A870G (Pro241Pro), with breast cancer risk alone and in combination with estrogen metabolism enzyme catechol-o-methyltransferase (COMT) gene polymorphism Met108/158Val was examined among 1956 breast cancer cases and 1406 controls from two populations from Finland and Ontario, Canada. The CCND1 high enzymatic activity allele A was associated with increased breast cancer risk (OR 1.3 in Ontario and 1.4 in Finland), and the interaction of the high-activity alleles of CCND1 and COMT conferred an even higher risk (OR 2.2 and 1.7, respectively). These results show that CCND1 and COMT act synergistically to contribute to breast cancer progression and that individual risk for breast cancer can be altered by the combined effect of polymorphisms with low-penetrance alleles.

Cyclin B1 expression was studied among 1348 breast cancers on TMA. High cyclin B1 expression was associated with aggressive breast cancer features, but had an independent impact on survival. Besides tumor size and nodal status, cyclin B1 was the only independent predictor of poor metastasis-free survival among chemotherapy-naïve patients. This is the largest study by far investigating the prognostic role of cyclin B1 in breast cancer, and the results suggest that cyclin B1 immunohistochemistry is a method that could easily be adapted for routine use and is an independent prognostic factor, adding specificity to prognostic evaluation conducted with traditional markers.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred in the text by Roman numerals I-V:

I. Aaltonen K, Ahlin C, Amini RM, Salonen L, Fjällskog ML, Heikkilä P, Nevanlinna H, Blomqvist C. Reliability of cyclin A assessment on tissue microarrays in breast cancer compared to conventional histological slides. *Br J Cancer* 2006;94:1697-702.

II. Aaltonen K, Blomqvist C, Amini RM, Eerola H, Aittomäki K, Heikkilä P, Nevanlinna H. Familial breast cancers without mutations in BRCA1 or BRCA2 have low cyclin E and high cyclin D1 in contrast to cancers in BRCA mutation carriers. *Clin Cancer Res* 2008;14:1976-83.

III. Onay UV, Aaltonen K, Briollais L, Knight JA, Pabalan N, Kilpivaara O, Andrulis IL, Blomqvist C, Nevanlinna H, Ozcelik H. Combined effect of CCND1 and COMT polymorphisms and increased breast cancer risk. *BMC Cancer* 2008;8:6.

IV. Aaltonen K, Amini RM, Landberg G, Eerola H, Aittomäki K, Heikkilä P, Nevanlinna H, Blomqvist C. Cyclin D1 expression is associated with poor prognostic features in estrogen receptor positive breast cancer. *Breast Cancer Res Treat*, epub 1 February 2008.

V. Aaltonen K, Amini RM, Heikkilä P, Aittomäki K, Tamminen A, Nevanlinna H, Blomqvist C. High cyclin B1 expression is an independent marker of poor metastasis free and overall survival in breast cancer. Submitted.

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ABBREVIATIONS

A	alanine
BRCA1	breast cancer gene 1
BRCA2	breast cancer gene 2
BRIP1 (BACH1)	BRCA1 interacting protein 1 (BRCA1-associated C-terminal helicase)
CA-125	cancer antigen 125
CASP8	caspase 8
CCND1	cyclin D1 gene
CCNE	cyclin E gene
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CHD5	chromodomain helicase DNA binding domain 5
CHEK2	cell cycle checkpoint kinase 2
CI	confidence interval
CIP/KIP	cyclin-dependent kinase inhibitory protein/kinase inhibitor protein
CISH	chromogenic in situ hybridization
CK	cytokeratin
CKI	cyclin-dependent kinase inhibitor
COMT	catechol-o-methyl transferase
CT	chemotherapy
CYP	cytochrome P450
DNA	deoxyribonucleic acid
E2F	E2 promoter binding factor
ER	estrogen receptor
ET	endocrine treatment
G	guanine
G0 phase	quiescent phase of the cell cycle
G1 phase	gap 1, cell cycle phase before S phase
G2 phase	gap 2, cell cycle phase before mitosis
GSTM1	glutathione S-transferase M1
HER2 (ERBB2)	human epidermal growth factor receptor 2
IHC	immunohistochemistry
INK4	inhibitor of kinase 4
LKB1 (STK11)	serine/threonine protein kinase
LMW	low molecular weight
LOH	loss of heterozygosity
M	distant metastases
M, M phase	mitosis
Met	methyline
MFS	metastasis-free survival
MRI	magnetic resonance imaging
N	regional lymph nodes

OFBCR	Ontario Familial Breast Cancer Registry
OR	odds ratio
OS	overall survival
p53	tumor protein 53
PALB2	partner and localizer of BRCA2
PR	progesterone receptor
pRb	retinoblastoma protein
Pro	proline
PTEN	phosphatase and tensin homolog
RFS	recurrence-free survival
RNA	ribonucleic acid
RR	relative risk
S phase	DNA synthesis and replication phase of cell cycle
SNP	single-nucleotide polymorphism
SPF	S phase fraction
T	primary tumor size
TGFB1	transforming growth factor beta 1
TMA	tissue microarray
Val	valine

1 INTRODUCTION

Breast cancer is a heterogeneous disease with varying biological characteristics, aggressiveness, and prognosis. Breast cancer carcinogenesis is only partly understood, and although subclassification has proceeded, many aspects of tumorigenesis and molecular mechanisms are still unclear.

Family history is one of the strongest predisposing factors for breast cancer. Known high- and low-penetrance risk genes explain only 25% of familial predisposition for breast cancer. At least some of the excess familial risk may be explained by commonly occurring low-penetrance polymorphisms that either alone or interacting with other polymorphisms or environmental factors modify the individual risk for breast cancer. Tumors of familial breast cancer patients not attributable to mutations in known high-penetrance genes BRCA1 or BRCA2 have not been extensively characterized but they seem to be of lower grade than BRCA1, BRCA2, or sporadic tumors and more often estrogen receptor- (ER) and progesterone receptor- (PR) negative and p53-positive than sporadic tumors. Learning more about the characteristics of tumors in this patient group can shed light on the pathogenesis and genetic background of familial tumors not attributable to BRCA1 or BRCA2 mutations as well as to help to identify them from mutation-positive familial breast cancers.

Due to earlier diagnosis and effective adjuvant treatment, breast cancer prognosis has improved and more than 85% of breast cancer patients are alive five years after diagnosis. A small number of breast cancers still have a poor prognosis and despite adjuvant treatment metastasize and lead to death even shortly after diagnosis. Breast cancer prognosis is traditionally defined by tumor size, tumor grade, and nodal status. Of biological markers, hormone receptor and human epidermal growth factor receptor 2 (HER2) status affect prognosis and responsiveness to treatment. A high proliferation rate seems to be associated with poor prognosis, but uncertainty remains about the optimal methods and cut-offs for measuring proliferation. Although adjuvant chemotherapy and endocrine treatment are generally well tolerated, they can cause acute side-effects and harmful long-term effects, and thus, overtreatment should be avoided. New prognostic markers to improve prognostic assessment and adjuvant treatment decisions are needed.

Cyclins are proteins that regulate the cell cycle. Cyclin expression oscillates during the cell cycle and is under strict control. In cancer, cyclin expression often becomes deregulated. Cyclin overexpression can lead to uncontrolled cell division and proliferation and be one of the crucial mechanisms in cancer development and progression. Cyclin expression can be detected by immunohistochemical staining and can provide information on biological characteristics and prognosis of the tumor. This study focuses on bringing new insights into the roles of cell cycle regulator proteins cyclins in breast cancer.

2 REVIEW OF THE LITERATURE

2.1 General aspects of breast cancer

2.1.1 Epidemiology

Breast cancer is the most common malignancy in women of industrialized countries. An estimated one million women worldwide are diagnosed each year with breast cancer, and it is the leading cause of cancer death. In Finland, more than 4000 new cases were diagnosed in 2006 (www.cancerregistry.fi). Of all female cancers, about one-third are breast cancers. Breast cancer affects primarily older women; only about 25% of breast cancers are diagnosed in women under 50 years of age (Brenner & Hakulinen 2004). The mean age at diagnosis is approximately 60 years. Known risk factors for breast cancer include family history, early menarche, nulliparity or late age at first birth, no history or short duration of breastfeeding, low number of children, late menopause, long-term use of postmenopausal hormone replacement therapy, benign proliferative breast disease e.g. atypical hyperplasia or sclerosing adenosis, carcinoma of the contralateral breast, obesity when postmenopausal, low physical activity, exposure to ionizing radiation, smoking, and alcohol abuse (reviewed in Oldenburg et al. 2007).

2.1.2 Carcinogenesis

Cancer is defined as abnormal and destructive growth of cells or tissue that is independent of an external growth signal. Malignant cancer cells do not require growth signals, are insensitive to negative growth inhibitory signals, are capable of avoiding apoptosis, have limitless proliferative capacity, possess angiogenesis, and are able to invade neighboring tissues and develop metastases. Cancer develops from a single cell, which after DNA damage begins to proliferate in an uncontrolled manner. Daughter cells acquire further DNA damage and receive a growth advantage.

Cancer is a genetic disease of somatic cells that develops after a series of mutations in a cell. DNA damage leading to carcinogenesis does not occur randomly in the genome, but affects genes essential to the control of cell growth and proliferation. These mutations are

either gain-of-function mutations that activate oncogenes encoding proteins needed in cell growth, such as transcription and growth factors, or loss-of-function mutations that inactivate tumor suppressor genes inhibiting cell cycle-, proliferation-, and apoptosis-associated events (Kinzler & Vogelstein 1997 & 1998). Tumor suppressor genes can be described as gatekeepers, caretakers, or landscapers. Gatekeepers exert functions that directly inhibit cell growth or promote death by apoptosis. Caretaker genes control the genomic integrity, and landscapers regulate the cellular microenvironment.

Breast carcinogenesis is a multi-step process in which normal breast epithelium evolves via hyperplasia and carcinoma in situ into an invasive cancer, which eventually can disseminate via the lymphatic and vascular systems to form distant metastases (Beckmann et al. 1997). Each of these steps is thought to correlate with one or more mutations in either oncogenes or tumor suppressor genes. In sporadic breast cancer, these mutations are acquired and occur in somatic cells without an underlying germline mutation. Essential early events in sporadic breast carcinogenesis are mutational activation of such oncogenes as c-myc, cyclin D1 gene (CCND1), or HER2 (Nass et al. 1997, Ormandy et al. 2003, Owens et al. 2004). Overexpression of ER is also frequently observed in early breast cancer (Yager et al. 2006). After first events, the tumor progression can proceed through different pathways, and breast carcinogenesis may be different in tumors with different histopathological features (Bürger et al. 1999 & 2000).

Oncogenes that commonly obtain somatic mutations in breast cancer include c-myc, CCND1, p53, HER2, and PTEN. Recent advances in sequencing and bioinformatics have made it possible to analyze the somatic mutations of a cancer on a large scale. These large-scale cancer genome studies have revealed that the amount of mutations in breast cancer development may be higher than previously thought (Sjöblom et al. 2006, Wood et al. 2007). In a typical breast cancer, as many as 80 mutations can be present at the time of clinical diagnosis, but less than 15 are usually considered essential for tumor initiation and progression (Wood et al. 2007). Mutated genes in the cancer genome include some very often mutated genes, as well as a larger amount of infrequently mutated genes. The breast cancer genome seems to contain different mutated genes than other cancers. Of the large amount of mutated genes, many share functions in the same pathways or their protein products interact; thus, the number of interrupted pathways may be lower. Large-scale

breast cancer genome analyses suggest new potential oncogenes or tumor suppressor genes, e.g. NF- κ B pathway kinase IKBKE or chromodomain helicase DNA binding domain 5 (CHD5), in addition to the traditionally known breast cancer oncogenes (Wood et al. 2007).

Estrogen has a crucial role in breast carcinogenesis. Increased risk for breast cancer is associated with an elevated blood level of endogenous estrogen as well as with exposure to exogenous estrogen and progestin through hormone replacement therapy (Oldenburg et al. 2007). In experimental animals, estrogen treatment has led to the development of mammary tumors (Shull et al. 1997). Recent evidence suggests that estrogen may play a role even in the development and progression of ER- negative breast cancer, and operate by mechanisms other than by binding the ER (Gupta et al. 2007). Estrogen seems to be capable of contributing to all phases of the carcinogenic pathway, although the exact mechanisms are not fully understood. The toxic catechol estrogen metabolites are hypothesized to cause direct DNA damage, and ER-mediated genomic and non-genomic signaling may induce cell proliferation and inhibit apoptosis (Yager et al. 2006).

In hereditary breast cancer, one tumor suppressor gene has a germline pathological mutation. Inactivation of the second allele, in addition to the germline mutation present in the first allele, is an early event in the pathway towards cancer. This “two-hit” model of carcinogenesis has originally been suggested by Knudson (1971). Mutation of the second allele may result from damage by endogenous toxins or occur by chance during DNA replication, leading to loss of function of the tumor suppressor. Loss of heterozygosity (LOH) is a common way to lose the wild-type allele. A mutated tumor suppressor gene can also cause cancer by mechanisms that do not require losing both alleles: by dominant-negative effect, which means that the mutated allele disturbs the function of the normal allele (Brachman et al. 1996, Kwabi-Addo et al. 2001, Chevenix-Trench et al. 2002), or by haploinsufficiency, i.e. the normal allele is insufficient for the proper function of the gene (Fero et al. 1998, Fodde & Smits 2001).

Current understanding of differences in oncogenic pathways between hereditary and sporadic breast cancers is far from complete, but in the former tumorigenesis appears to most often start from the inactivation of two alleles of a tumor suppressor gene, while in

the latter the early event is more frequently genomic amplification of only one allele of an oncogene (Kenemans et al. 2004).

2.1.3 Tumor features and histopathological classification

Practically all breast cancers are adenocarcinomas derived from glandular tissue (Berg & Hutter 1995). The general architecture of the tumor defines the histological type. The main subtypes are ductal and lobular carcinomas. Invasive ductal carcinoma derives from epithelial cells lining the ducts and is the most common type, accounting for approximately 70% of breast cancers. Invasive lobular carcinoma is the second most common type (10-20%). Lobular carcinoma is formed from acinus cells located in terminal ducts. Lobular carcinoma has a slightly better prognosis than ductal carcinoma but the risk for contralateral breast cancer is higher than with other subtypes. Lobular carcinoma metastasizes into serosal surfaces, cerebrospinal fluid, bone marrow, uterus, and ovaries more often than the other subtypes. Medullary carcinoma accounts for 1-7% of all breast cancers and is characterized by solid sheets of large cells with prominent nucleoli and frequent mitosis, and a noninfiltrative (pushing) margin. Medullary carcinoma has a somewhat better prognosis than ductal or lobular carcinoma. Mucinous and tubular carcinomas represent 1-2% of breast cancers each. Mucinous carcinoma is a slowly growing carcinoma occurring in older women, and tubular carcinoma is a well-differentiated carcinoma with an excellent prognosis. Papillary carcinoma accounts for less than 1% of breast cancers.

Tumor histological grade defines the differentiation of the tumor cells. Grading is performed using the method by Bloom and Richardson (1957), modified by Elston and Ellis (1991). The histological grade is based on three features; tubule formation, nuclear pleomorphism, and mitotic count, and divides breast tumors into three categories. Grade I tumors are well, grade II tumors moderately, and grade III tumors poorly differentiated.

Breast cancer staging is performed by TNM classification that describes the size and extent of the tumor (T), involvement of local lymph nodes (N), and distant metastases (M). The TNM classification was accepted and is being updated by the International Union Against Cancer (UICC) (<http://www.uicc.org/>).

The routine pathological classification of a breast tumor also defines the lymphatic or vascular channel invasion, infiltration into skin, presence and amount of estrogen and progesterone receptors, and expression of HER2 protein, and presence of HER2 gene amplification. The tumor is considered hormone receptor-positive if 10% or more of the cells are stained. Approximately 80% of breast cancers are ER-positive, and most of these tumors are also PR-positive. The proportion of hormone receptor-positive cancers seems to be rising. Hormone receptor-positive tumors are sensitive to endocrine treatment. HER2 gene amplification leading to immunohistochemical overexpression is detected in around 15-20% of breast cancers (Slamon et al. 1989, Ravdin & Chamness 1995, Owens et al. 2004). Tumors with positive HER2 status relapse more often and more rapidly than HER2-negative tumors (Joensuu et al. 2003). The proliferation rate of tumor cells and the expression of the tumor suppressor protein p53 expression are also frequently determined.

Table 1 *TNM classification of breast cancer (modified from UICC 2002)*

Primary tumor (T)	
<i>pTis</i>	carcinoma in situ
<i>pT1</i>	≤2.0 cm
T1mic	≤0.1 cm
T1a	>0.1 cm but ≤0.5 cm
T1b	>0.5 cm but ≤1.0 cm
T1c	>1.0 cm but ≤2.0 cm
<i>pT2</i>	>2.0 cm but ≤5.0 cm
<i>pT3</i>	>5.0 cm
<i>pT4</i>	direct extension to skin or chest wall
T4a	extension to chest wall (not pectoralis muscle)
T4b	edema or ulceration of the skin, satellite skin nodules
T4c	both T4a and T4b
T4d	inflammatory carcinoma
Regional lymph nodes (N)	
<i>pN0</i>	no regional lymph node metastasis
<i>pN1</i>	metastasis in movable ipsilateral axillary lymph node(s)
N1mi	micrometastasis >0.2 mm but ≤2 mm
N1a	metastasis in 1-3 axillary lymph nodes
N1b	micrometastasis in internal mammary nodes detected by sentinel lymph node dissection
N1c	metastasis in 1-3 axillary lymph nodes and micrometastasis in internal mammary nodes detected by sentinel lymph node dissection
<i>pN2</i>	
N2a	metastases in 4-9 axillary lymph nodes
N2b	metastasis in clinically apparent internal mammary lymph nodes in the absence of axillary lymph node metastases
<i>pN3</i>	
N3a	metastasis in 10 or more axillary lymph nodes or in infraclavicular lymph nodes
N3b	metastasis in internal mammary lymph nodes and axillary lymph nodes, or in 3 or more axillary lymph nodes and micrometastasis in internal mammary lymph nodes detected in sentinel lymph node dissection
N3c	metastasis in ipsilateral supraclavicular lymph nodes
Distant metastases (M)	
<i>M0</i>	no distant metastasis
<i>M1</i>	distant metastasis

2.1.4 Molecular classification

Recently, complementary DNA (cDNA) microarray-based gene expression profiling has brought a new classification of invasive breast cancers (Perou et al. 2000, Sørlie et al. 2001 & 2003). Five distinct breast cancer subtypes associated with different clinical outcomes can be identified. Luminal A and luminal B subtype tumors are ER-positive and share features with luminal epithelial cells that arise from the inner layer of duct lining. Luminal A tumors have the highest expression of ER gene, and luminal B tumors show low to moderate expression of luminal specific genes, including the ER cluster. The third

subtype is ER-negative and overexpresses HER2 protein. The fourth subtype resembles normal breast cells and is characterized by high expression of basal epithelial and low expression of luminal epithelial genes. The fifth subtype is basal-like, with ER-, PR-, and HER2-negative tumors that express basal cytokeratins (CK) 5/6 and 17. These tumors show characteristics of myoepithelial cells from the outer (basal) layer of breast ducts. Basal-like tumors have the highest proliferation rates and a poor prognosis (Foulkes et al. 2004). Overall, little is known about the development of aggressive basal-like tumors. Whether ER is actually involved in the pathogenesis of these tumors is uncertain.

2.1.5 Prognostic aspects

Although breast cancer incidence has been on the rise, the mortality has not. Today, more than 85% of breast cancer patients are alive five years after diagnosis (www.cancerregistry.fi), and an estimated 75% will survive. Risk for recurrence is highest during the first five years after diagnosis, but breast cancer can metastasize even 15-20 years after diagnosis. The improved prognosis is due to earlier diagnosis and more effective adjuvant treatment (Berry et al. 2005). The most important prognostic factors in breast cancer are nodal status, tumor size, and histological grade. These traditional prognostic factors are used to identify breast cancer patients at high risk for recurrence who will potentially benefit from adjuvant chemotherapy (Singletary et al. 2002). Young age (<35 years) at diagnosis is an independent marker of poor prognosis (Dubsky et al. 2002). Of biological markers, negative hormone receptor status and HER2 gene amplification predict poor outcome (Isaacs et al. 2001, Joensuu et al. 2003, Harris et al. 2007). Lymphatic or vascular channel invasion and high proliferation rate (Dettmar et al. 1997, Bryant et al. 1998, Jansen et al. 1998, Clahsen et al. 1999) are also associated with poor breast cancer survival. Although HER2 positivity alone predicts poor outcome, the so-called triple-negative (ER, PR, HER2) breast cancers seem to have a highly aggressive clinical course with short recurrence-free periods and poor overall survival (Dent et al. 2007). A large number of additional prognostic tumor markers have been published, but for many markers the prognostic value has not been shown in subsequent studies, and thus the true prognostic value remains unclear, and these markers have not been adapted for routine use. cDNA studies have identified poor prognostic gene expression profiles that

may allow more specific prognostic evaluation than by immunohistochemical methods (van't Veer et al. 2002, van de Vijver et al. 2002, Paik et al. 2004). At the moment, how much these methods supplement prognostic evaluation and treatment decisions in clinical use is uncertain.

Although breast cancer prognosis has improved and can be considered good, a small proportion of breast cancers behave aggressively and despite adjuvant treatment metastasize and lead to death shortly after diagnosis. Finding new prognostic markers could help in identifying these tumors more accurately and possibly uncover novel strategies for developing future targeted therapies. More precise prognostic evaluation can also help in making treatment decisions and avoiding overtreatment, and decreasing the exposure of patients who do not actually benefit from treatment to the side-effects of adjuvant therapies.

Table 2 *Definition of risk for recurrence by tumor markers. Adapted from the St. Gallen consensus meeting recommendations (Goldhirsch et al. 2007).*

Low-risk tumors

Node-negative and all of the following features:

pT ≤2cm, grade 1, absence of vascular invasion, ER- and/or PR-positive, absence of HER2 overexpression or gene amplification, age ≤35 years

Intermediate-risk tumors

Node-negative and at least one of the following features:

pT >2cm, or grade 2-3, or presence of vascular invasion, or ER- or PR-negative, or HER2 overexpression or gene amplification, or age ≤35 years

Node-positive (1-3 nodes involved) and:

ER- and/or PR-positive and absence of HER2 overexpression or gene amplification

High-risk tumors

Node-positive (1-3 nodes involved) and

ER- or PR-negative, or HER2 overexpression or gene amplification

Node-positive (4 or more nodes involved)

2.1.6 Adjuvant treatment

Patients treated with local resection or those who have undergone mastectomy and have a high risk for recurrence (tumor size more than 5 cm or more than three regional lymph nodes involved) are recommended to receive postoperative radiotherapy. Radiotherapy

significantly diminishes the risk of local relapse (Clark et al. 1996, Fisher et al. 2002, EBCTCG 2005).

The majority of breast cancer patients nowadays are also advised to have adjuvant chemotherapy and/or endocrine treatment. Only patients considered to have a small risk (<10%) of recurrence (Goldhirsch et al. 2007) do not seem to benefit from adjuvant treatment. Recurrence risk and the need for adjuvant treatment are defined by the presence of the adverse prognostic factors described above; and these recommendations are updated regularly. Adjuvant treatment significantly reduces the risk of recurrence and improves the survival of moderate- and high-risk breast cancer patients, but also exposes patients to harmful side-effects. A future challenge is to tailor the treatment based on the biological profile of the tumor to identify patients who will optimally benefit from each regimen.

Table 3 *Adjuvant treatment for early invasive breast cancer. Modified from the St. Gallen consensus meeting recommendations (Goldhirsch et al. 2007).*

Recurrence risk	Highly endocrine-responsive*	Incompletely endocrine-responsive**	Endocrine-non-responsive***
Small	Endocrine treatment	Endocrine treatment	No treatment
Medium	Endocrine treatment, or chemotherapy and endocrine treatment and trastuzumab if HER2-positive tumor	Chemotherapy and endocrine treatment and trastuzumab if HER2-positive tumor	Chemotherapy and trastuzumab if HER2-positive tumor
High	Chemotherapy and endocrine treatment and trastuzumab if HER2-positive tumor	Chemotherapy and endocrine treatment and trastuzumab if HER2-positive tumor	Chemotherapy and trastuzumab if HER2-positive tumor

* hormone receptor-positive breast cancer

** borderline hormone receptor positivity, or negative progesterone receptor

*** hormone receptor-negative breast cancer

Patients with hormone receptor-positive tumors are offered endocrine therapy either alone or in combination with chemotherapy (Goldhirsch et al. 2007). Endocrine treatment consists of tamoxifen or aromatase inhibitors (anastrozole, letrozole, and exemestane), alone or in combination. Tamoxifen treatment for five years after diagnosis diminishes the risk of recurrence by 50% and of breast cancer-related death by 31% (EBCTCG 2005). Treatment with aromatase inhibitors alone for five years, changing tamoxifen to aromatase inhibitors after 2-3 years, or continuing endocrine treatment with aromatase inhibitors for 2-5 years after five years of tamoxifen improves clinical outcome among high-risk

postmenopausal patients (Goss et al. 2003 & 2007, Coombes et al. 2004, Howell et al. 2005, Coates et al. 2007, Kaufmann et al. 2007, Forbes et al. 2008).

Adjuvant polychemotherapy containing an anthracycline regimen improves the survival of premenopausal high- or medium-risk patients by ~45% and of postmenopausal patients by ~26% (EBCTCG 2005). Adjuvant chemotherapy with taxanes further improves outcome of the patients at the highest risk for recurrence but, is also more toxic. Taxane treatment improves recurrence-free survival of lymph node-positive patients by 17% and overall survival by 15% (De Laurentiis et al. 2008). At the moment, no predictive tumor features for chemotherapy response are in standard use.

Patients who have breast cancer showing HER2 gene amplification benefit from adjuvant treatment with HER2 protein targeting antibody trastuzumab in combination with chemotherapy, and in the case of endocrine-responsive tumor, with endocrine treatment (Piccart-Gebhart et al. 2005, Romond et al. 2005, Joensuu et al. 2006, Smith et al. 2007). Trastuzumab seems to prevent half of recurrences of these patients.

2.2 Familial background for breast cancer

Family history is considered one of the strongest predisposing factors for breast cancer. First-degree relatives of a breast cancer patient have an approximately twofold risk for breast cancer compared with the general population. Genetic factors may play a role in up to 30% of breast cancers, but known high-risk susceptibility genes explain only 20-30% of hereditary and 5% of all breast cancer incidences (Lichtenstein et al. 2000).

2.2.1 Breast cancer risk in cancer predisposition syndromes

Certain rare autosomal-dominant cancer predisposition syndromes cause elevated breast cancer risk in addition to other cancer risks. Carrier women of p53 mutations in Li-Fraumeni syndrome have a 28-56% risk for breast cancer by the age of 45 years (Chompret et al. 2000). Cowden syndrome is caused by tumor suppressor gene PTEN mutations and confers a 20-50% lifetime risk for breast cancer (Eng 2003). Women with Peutz-Jeghers syndrome (LKB1 mutations) have up to 50% risk for breast cancer by the

age of 70 years (Hearle et al. 2006). These rare cancer predisposition syndromes explain less than 1% of all familial breast cancers.

2.2.2 High-penetrance susceptibility genes BRCA1 and BRCA2

The most important predisposing high-penetrance genes are BRCA1 on 17q21 and BRCA2 on 13q12-13 (Miki et al. 1994, Wooster et al. 1995). Numerous mutations have been reported throughout the coding regions of these large genes. BRCA1 and BRCA2 function in DNA repair (Yoshida & Miki 2004, Zhang & Powell 2005) and act as classical tumor suppressor genes. LOH is a common phenomenon in BRCA1/2-related tumors. Women carrying germline BRCA1 or BRCA2 mutation have a high lifetime risk for breast (50-90% and 40-80%, respectively) and ovarian cancers (20-50% and 10-20%, respectively) (Antoniou et al. 2003). Mutations in BRCA1 and BRCA2 explain ~20% of familial breast cancers in Finland (Vehmanen et al. 1997, Vahteristo et al. 2001, Hartikainen et al. 2007).

2.2.3 Low-penetrance susceptibility genes

Checkpoint kinase 2 (CHEK2) is a signal transducer that plays a crucial role in response to DNA damage. CHEK2 1100delC variant is the first recognized low- or moderate-penetrance susceptibility variant in breast cancer, conferring an approximately twofold increased risk (Meijers-Heijboer et al. 2002, Vahteristo et al. 2002). Other variants in CHEK2 have also been suggested in breast cancer predisposition, and at least the I157T seems to be associated with increased breast cancer risk (Cybulski et al. 2004, Kilpivaara et al. 2004). The BRCA2-binding protein PALB2 gene has recently been identified as a new susceptibility gene with a 2- to 4-fold hereditary risk for breast cancer (Rahman et al. 2006, Erkkö et al. 2007). BRIP1 (BACH1) is a BRCA1-associated helicase that participates in DNA double-strand break repair with BRCA1. BRIP1 germline mutations have also been shown to be associated with an approximately twofold increased breast cancer risk (Seal et al. 2006). These low- or moderate-penetrance breast cancer susceptibility genes are estimated to account for ~5% of hereditary breast cancers.

2.2.4 Familial breast cancer predisposition not explained by known susceptibility genes

Even considering all known high- and low-penetrance breast cancer susceptibility genes, the genetic background of ~70-75% of familial breast cancers remains unknown. Studies are underway to discover new breast cancer susceptibility genes. A polygenic model in breast cancer susceptibility has also been suggested and can explain some of the residual familial breast cancer aggregation (Antoniou et al. 2002, Pharoah et al. 2002, Antoniou & Easton 2006). Low-penetrance variants that occur commonly in the general population may individually confer a moderate breast cancer risk, but have a multiplicative effect when combined. These low-penetrance variants may also interact with environmental risk factors such as estrogen exposure. The strongest evidence for common breast cancer susceptibility alleles by far comes from a large collaboration study and implies that the D302H variant in the caspase 8 (CASP8) gene and the L10P variant in the transforming growth factor beta 1 (TGFB1) gene are associated with breast cancer risk and may explain 0.3% and 0.2%, respectively, of the excess familial breast cancer risk in European populations (Cox et al. 2007). Furthermore, a large genome-wide association study has recently found five novel independent loci with strong and consistent breast cancer associations (Easton et al. 2007). Genes and variants causing this breast cancer risk are subjects for further research.

2.2.4.1 COMT Met108/158Val polymorphism

Estrogen has a critical role in breast cancer development. Estrogen stimulates transcription of genes essential in cell proliferation through estrogen receptor and is suggested to cause DNA damage via catechol estrogen metabolites (Zhu & Conney 1998). A number of polymorphisms have been depicted in enzymes involved in estrogen synthesis or metabolism. Such variants include catechol-o-methyltransferase (COMT), cytochrome P-450 (CYP) 17, 19, 1A1, and 1B1, and glutathione S-transferase M1 (GSTM1) genes. No significant association with breast cancer risk in subsequent materials has been found for any of these variants, but the combined effect of some of the variants may confer breast cancer risk or individual vulnerability to exogenous estrogen exposure (Yager et al. 2006).

Catechol-o-methyltransferase (COMT) is a phase two enzyme in estrogen metabolism that catalyzes these toxic catechol estrogen metabolites into methoxyestrogens (Zhu 2002). Detoxification of catechol estrogens happens mainly in the liver, but also in peripheral tissues such as the breast. COMT is expressed in brain, liver, kidney, and peripheral tissues including the epithelium of the ducti and lobuli of normal breasts. In breast tumor tissue, COMT expression is elevated compared with in normal breasts (Tenhunen et al. 1999). COMT activity varies among individuals (Weinshilboum & Dunnette 1981), and COMT gene has a commonly occurring A to G single-nucleotide polymorphism (SNP) (Met108/158Val) at position 1947 (rs 4680). This polymorphism produces two different alleles of COMT: Met and Val. The Val allele has been suggested to have a 3- to 4-fold higher enzymatic activity than the Met allele (Lotta et al. 1995, Lachman et al. 1996).

2.2.4.2 COMT Met108/158Val polymorphism and breast cancer risk

The COMT Met108/158Val polymorphism has a role in some neurological and psychiatric diseases, and its association with risk for cancer has been studied widely. Higher risk for breast cancer has been suggested for MetVal and ValVal genotypes (Lavigne et al. 1997, Ahsan et al. 2004, Sazci et al. 2004, Gaudet et al. 2006), although all studies have not confirmed the association (Millikan et al. 1998, Thompson et al. 1998, Mitrinen et al. 2001, Wedren et al. 2003, Onay et al. 2006, Akisik et al. 2007).

2.2.4.3 CCND1 G870A polymorphism

Uncontrolled cell proliferation is one of the hallmarks of cancer. The cell cycle is the machinery that governs proliferation and growth of cells, thus, alteration in genes regulating the cell cycle could predispose to cancer. Cyclin D1 has a critical role in control of cell cycle G1 to S phase transition. Cyclin D1 coding gene CCND1 has a commonly occurring G to A polymorphism (G870A, Pro241Pro) at position 6962 (rs 603965) in exon 4. The frequency of the A allele in the Caucasian population is approximately 44% and of the G allele 56%, but frequencies have varied in different populations (Sanyal et al. 2004). This polymorphism produces two alternatively spliced forms of a transcript with varying stability and enzymatic activity. The protein product of the CCND1 A allele has higher

enzymatic activity and is hypothesized to be more stable than the product of the G allele (Betticher et al. 1995).

2.2.4.4 CCND1 G870A polymorphism and cancer risk

The CCND1 G870A A allele has been reported to be associated with increased risk for several cancers, including colorectal, esophageal, cardiac, bladder, head and neck, lung, prostatic, and renal cell carcinomas as well as leukemia (reviewed in Knudsen et al. 2006). The results have not been entirely consistent since some studies have shown no risk and increased risk for cancer has even been reported for the GG genotype. Thus, the effect of this polymorphism may vary in distinct tumor types, although differences in study designs can also explain some of the discrepancies. The highest risks have, however, been reported in most studies for the AA genotype. The relative risks have typically been modest, which can in part be expected for a common allele such as the A allele in CCND1 polymorphism. The cancer risk with this low-penetrance variant could be modified by polymorphisms in other genes or environmental factors (Buch et al. 2005, Shu et al. 2005).

2.2.4.5 CCND1 G870A polymorphism in breast cancer

Four studies have evaluated the association of CCND1 polymorphism with breast cancer risk (Griew et al. 2003, Krippel et al. 2003, Försti et al. 2004, Shu et al. 2005). None of these showed a significantly increased breast cancer risk; the patient samples in these studies were, however, rather small (200-500 breast cancer cases). Nor has any association between the CCND1 polymorphism and specific clinicopathologic features of breast cancers been reported.

2.2.4.6 Interaction of CCND1 and COMT

CCND1 and COMT polymorphisms share functions in the estrogen pathway. Negative feedback by the COMT high-activity allele results in higher estrogen level, which may in turn increase CCND1 expression. If the CCND1 high activity allele was more stable, the combination of high enzymatic alleles of CCND1 and COMT could be thought to lead to

enhanced proliferation. A study investigating the interactions of different SNPs in genes involved in major cancer pathways suggested a significant association with elevated breast cancer risk for the CCND1 G870A and COMT Met108/158Val polymorphism interaction (Onay et al. 2006). Thus, the interaction of these common variants was suggested to play a role in individual breast cancer risk.

2.2.5 Histopathological features of familial breast cancer

Breast cancers of BRCA1 carriers differ from sporadic and familial non-BRCA1/2 cancers. BRCA1-associated cancers seem to be of higher grade, more often ER-, PR-, and HER2-negative, p53-positive, aneuploid, of medullary histology, and diagnosed at a younger age than sporadic cancers (Eisinger et al. 1996, Breast Cancer Linkage Consortium 1997, Jóhansson et al. 1997, Armes et al. 1998, Noguchi et al. 1999, Lakhani et al. 2002, Eerola et al. 2005). Bilateral cancer is common in BRCA1 carriers. Expression of cell cycle regulator cyclin E has been higher and cyclin D1 expression lower than among sporadic cancers (Osin et al. 1998, Armes et al. 1999, Vaziri et al. 2001, Palacios et al. 2005), and also higher expression of basal cytokeratins seems to characterize BRCA1 tumors as compared with sporadic ones (Lakhani et al. 2005). cDNA expression studies have shown that tumors of BRCA1 carriers are mostly of basal cell epithelial phenotype (Foulkes et al. 2003 & 2004, Sorlie et al. 2003).

BRCA2-associated breast cancers have shown a phenotype between the BRCA1-associated and sporadic tumors (Noguchi et al. 1999, Lakhani et al. 2002, van der Groep et al. 2006). In some studies, BRCA2-associated tumors have not differed from sporadic or familial non-BRCA1/2 tumors in terms of tumor grade, histology, or ER, PR, HER2, and p53 status (Noguchi et al. 1999, Lakhani et al. 2002, Eerola et al. 2005). Higher amount of ER positivity and higher grade among BRCA2 tumors than among unselected tumors has been suggested (Bane et al. 2007, Brekelmans et al. 2007). Bilateral cancer is more common also among BRCA2 carriers than sporadic breast cancer patients. cDNA studies have identified a distinct expression profile also for BRCA2 tumors (Hedenfalk et al. 2001). Cyclin E expression among BRCA2 tumors has been higher than among sporadic tumors but both higher and lower cyclin D1 expression has been reported (Osin et al. 1998, Armes et al. 1999, Palacios et al. 2005, van der Groep et al. 2006, Bane et al. 2007).

Breast cancers of familial non-BRCA1/2 patients are not as well characterized as BRCA1- or BRCA2-associated cancers. Three studies have investigated histopathological features of these tumors. They seem to be of lower grade than BRCA1, BRCA2, or sporadic tumors (Lakhani et al. 2000, Eerola et al. 2005), and the most common histological type is invasive ductal carcinoma (Lakhani et al. 2000, Palacios et al. 2003, Eerola et al. 2005). Higher proportions of ER- and PR-negative and p53-positive tumors have been described among familial non-BRCA1/2 tumors than among sporadic tumors (Palacios et al. 2003). Based on the expression profiles of 60 different genes, a cDNA study divided the non-BRCA1/2 tumors into two groups with varying aggressiveness (Hedenfalk et al. 2003).

2.2.6 Identification of high-risk families

The screening of BRCA1 and BRCA2 mutations is laborious and expensive. The selection of patients to whom genetic testing is offered should be as accurate as possible. Finding mutation-positive patients is essential since after the familial mutation has been recognized, predictive genetic testing can be offered to healthy family members at risk of having inherited the mutation. Women carrying germline BRCA mutation are recommended to undergo regular surveillance, including monthly breast self-examination, clinical breast examination every six months, and breast imaging (mammography and ultrasound, and breast MRI if possible) once a year. Gynecologic surveillance consists of transvaginal ultrasound examination and serum CA-125 tumor marker measurement every 6-12 months. Prophylactic operations (prophylactic mastectomy and/or salpingo-oophorectomy) can also be considered. Screening can help to detect cancers at an earlier stage, and prophylactic operations have been shown to reduce breast and ovarian cancer incidence (reviewed in Bermejo-Perez et al. 2007).

The probability of finding BRCA mutation is evaluated mainly based on family history. In a Finnish breast cancer material consisting of patients with three or more first- or second-degree relatives with breast or ovarian cancer (including the proband) or patients from families with two affected cases, the age of the youngest breast cancer patient and the number of patients with ovarian cancer in the family were the independent factors predicting BRCA mutation positivity (Vahteristo et al. 2001). A logistic model based on this data showed that 97% of mutation-positive families obtained a probability above 10%

for finding BRCA1 or BRCA2 mutation as compared with 43% of all families. The mean probability was 55% for mutation-positive and 11% for mutation-negative families. Several other models have been constructed to evaluate mutation probability. Incorporating tumor pathology data into probability models may improve mutation prediction models (Farshid et al. 2006, James et al. 2006).

2.3 Cell cycle

The human cell cycle is a firmly controlled machinery that regulates cell entry into mitosis and DNA replication, and thus, cell division. Cell cycle consists of different phases: G1 phase (preparation for DNA synthesis), S phase (DNA synthesis and replication), G2 phase (the second gap), and M phase (mitosis). G0 (quiescence) is a biochemically different resting phase, where cells are kept in the absence of proliferation stimuli, but from which cells upon growth factor stimulation can re-enter into the active cell cycle (Sherr 1996). As cells receive enough environmental proliferation stimuli, they move from G1 to S phase. This G1/S transition is considered irreversible (Sherr 1996). The G2 phase is important for DNA replication and for the correction of errors in replication.

Cyclin -dependent kinases (CDKs) regulate the cell cycle and changes between different phases. CDK levels remain relatively constant throughout the cell cycle, but they are activated and inactivated by periodic changes in levels of their binding proteins. Cyclins are cell cycle regulator proteins that activate cell cycle progression by binding to CDKs. Active cyclin-CDK complexes drive phosphorylation cascades throughout the cell cycle. Cyclin D1-CDK4/6 and cyclin E-CDK2 complexes act predominantly on G1 phase, cyclin A-CDK2 on S and G2 phases, and cyclin B1-CDK1 on G2 phase and mitosis. CDK inhibitors (CKIs) consist of large amount of inhibiting proteins, e.g. inhibitors of kinase 4 (INK4) group inhibitors CDKN2A (p16 and p14), CDKN2B (p15), CDKN2C (p18), and CDKN2D (p19), and the second group of CIP/KIP (cyclin-dependent kinase inhibitory protein/kinase inhibitor protein) inhibitors CDKN1A (p21) and CDKN1B (p27) (Sherr & Roberts 1999). INK4s bind to CDK4 and CDK6 to prevent their association with cyclin D1. CIP/KIP inhibitors do not affect cyclin binding, but form complexes with the G1/S transition CDKs. CKIs allow the cell cycle to be controlled at multiple levels.

The most important target of cyclin-CDK phosphorylation is the retinoblastoma protein (pRb) and other retinoblastoma family proteins such as p107 and p130. Active pRb represses the transcription factors essential for G1/S transition. Thus, pRb is a negative cell growth regulator. Upon stimuli of proliferation factors, pRb becomes inactivated and transcription factors needed for G1/S transition are activated. The pRb hyperphosphorylation disrupts its association with E2F, and activated E2F mediates transcriptional activation required for G1 progression. Cyclin D1-CDK4/6 complex is upregulated early after mitogenic stimuli and initiates the phosphorylation and inactivation of pRb (Baldin et al. 1993). Cyclin E-CDK2 complex becomes activated later and is necessary in pRb phosphorylation in late G1 phase as well as in events leading to DNA synthesis (Sheaff et al. 1997, reviewed in Caldon et al. 2006). Recent data suggests that also the cell cycle reentry from G0 to G1 phase is regulated by specific retinoblastoma family proteins in association with specific E2F transcription factors. Cyclin C/CDK3 complex controls the pRb suppression at this phase and seems to be critical in promoting cell exit from the quiescent G0 phase (Ren & Rollins 2004). The G0 to G1 transition, however, is incompletely understood. The factors controlling the activity and stability of the cyclin C/CDK3 complex and the Rb-independent mechanisms for cells to exit G0 remain to be elucidated.

During oncogenesis cell cycle control mechanisms become deregulated and cell division is uncontrolled (Sherr 1996). CDK deregulation is considered one of the key events in tumor cells gaining unstrained growth capacity. In breast cancer cells, the cell cycle machinery seems to be uncontrolled at multiple levels. Deregulation of G2/M phase transition is found, but the G1/S transition appears to be particularly important in many cell cycle events specific to breast cancer pathogenesis.

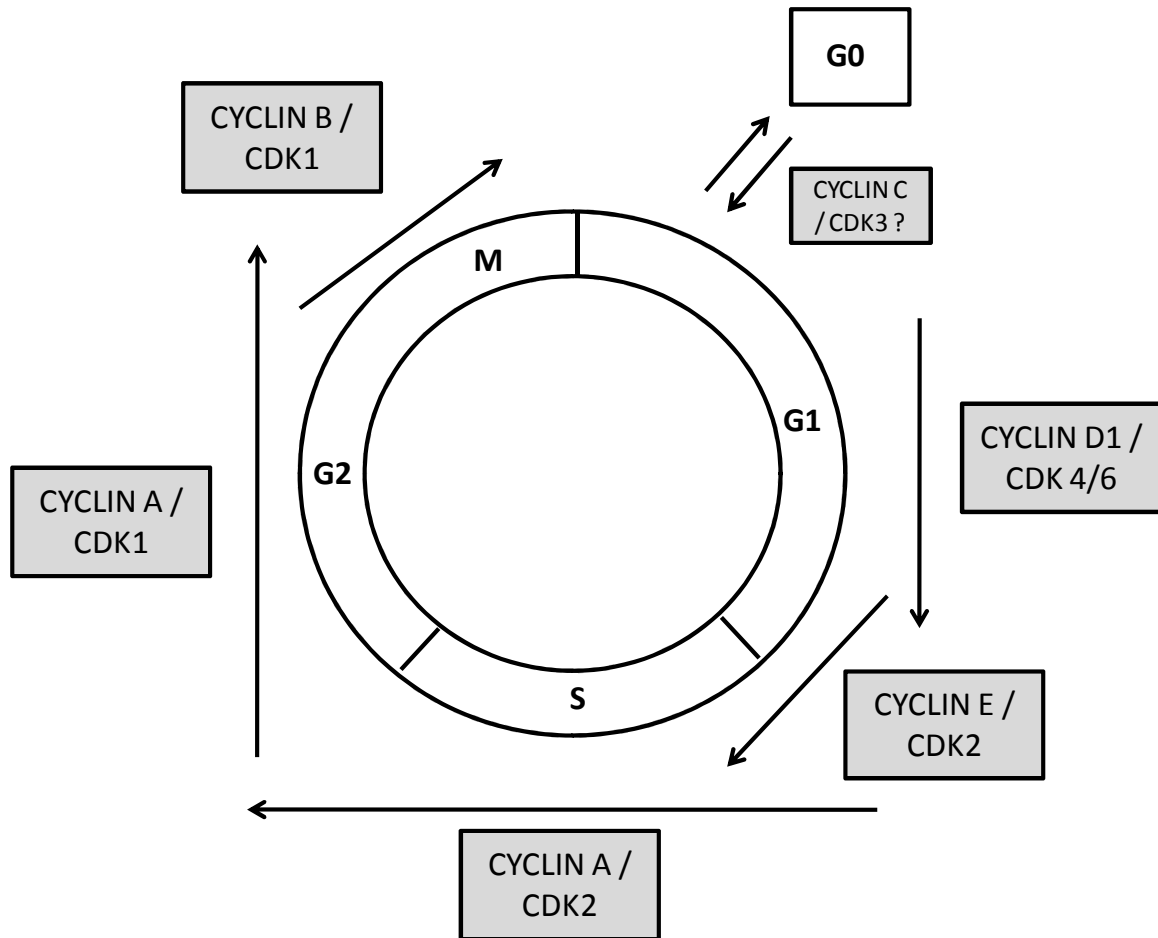


Figure 1 *The human cell cycle (adapted from Sherr 1996).*

2.3.1 Cyclin A

Cyclin A level in cells rises in early S phase and is essential for DNA replication and is also involved in G2/M transition (Zindy et al. 1992, Fan & Bertino 1997). Cyclin A/CDK2 complex regulates the pRb function in S and G2 phases and controls the initiation of DNA replication and timing of mitosis. Cyclin A overexpression is able to induce premature S phase induction, and removing cyclin A can block the S phase progression (Girard et al. 1991). In the mid-M phase, cyclin A is degraded and its level falls rapidly. Elevated cyclin A expression has been observed in several malignancies, including breast cancer (Bukholm et al. 2001), and is associated with poor cancer outcome at least in sarcomas and head and neck cancers (Huuhtanen et al. 1999a & 1999b, Saarilahti et al. 2003).

2.3.2 Cyclin A in breast cancer

Cyclin A overexpression is common in breast cancer. High cyclin A is more frequent among large, ER-negative, high-grade, cyclin E-, Ki67-, HER2-, and p53-positive and cyclin D1-negative tumors, as well as among tumors of younger patients. High cyclin A expression has been reported to be associated with poor prognosis in breast cancer (Bukholm et al. 2001, Michalides et al. 2002, Michels et al. 2003, Poikonen et al. 2005), although all studies have not confirmed this (Kühling et al. 2003, Rudolph et al. 2003). One explanation for the discrepancy in results might be the varying methods used to define cut-off values in different studies. Moreover, the study populations have differed. No association between cyclin A and poor prognosis was seen in patient material including only lymph node-negative patients, suggesting cyclin A may play a more important role in aggressive breast cancer and metastatic disease. A study aimed at determining the optimal cut-off value of cyclin A to identify highly proliferating tumors with poor prognosis suggested the optimal cut-off value to be around 8% (Ahlin et al. 2007). To assess optimal cut-off value, the material in this study was divided into two parts at cut-offs that were defined by splitting the material into deciles, and by calculating the relative risk (RR) for each of these cut-offs. The cut-off value of 8% corresponds to the 7th decile. In this study, cyclin A was an independent predictor of poor metastasis-free survival among patients not given adjuvant chemotherapy, but among patients receiving chemotherapy no significant association with prognosis was found. Based on these results, tumors with high cyclin A expression can be speculated to be particularly sensitive to chemotherapy.

2.3.3 Cyclin B1

Cyclin B1/CDK1 complex controls G2-M phase transition and is needed for initiation of mitosis (Pines & Hunter 1990). In cancer cells, cyclin B1 expression has been detected also in G1 phase (Shen et al. 2004). This unscheduled expression may lead to substrate phosphorylation regardless of the cell cycle phase, causing uncontrolled cell cycle progression, and be one of the mechanisms in carcinogenesis. The mechanism leading to this unscheduled expression remains to be elucidated; it can be due to disturbances either in synthesis or degradation of cyclin B1 protein. p53 protein has been shown to be able to

regulate the G2-M transition by increasing and decreasing cyclin B1 level by binding to its promoter site (Innocente et al. 1999). Disturbances in p53 control of cyclin B1 level may thus be one of the mechanisms leading to cyclin B1 overexpression, induced cell division, and transformation to malignancy. High cyclin B1 expression has been detected in several malignancies (Murakami et al. 1999, Soria et al. 2000, Nozoe et al. 2002, Takeno et al. 2002, Yoshida et al. 2004, Ikueworo et al. 2006). It has been associated with high tumor grade and advanced stage of disease as well as with poor prognosis in several cancers, including esophageal squamous cell (Murakami et al. 1999, Nozoe et al. 2002, Takeno et al. 2002), non-small cell lung (Soria et al. 2002, Yoshida et al. 2004), and renal cell cancers (Ikueworo et al. 2006).

2.3.4 Cyclin B1 in breast cancer

Cyclin B1 expression level is often high in breast cancer and has been shown to rise from benign and premalignant to advanced malignant breast lesions (Kawamoto et al. 1997). In breast cancer, the association of cyclin B1 with prognosis has thus far been evaluated in four rather small populations. In a study with 73 breast cancers, both nuclear and cytoplasmic expressions were independent predictors of poor relapse-free and overall survival (Winters et al. 2001). Cyclin B1 expression was not associated with tumor size, nodal status, grade, ER positivity, or p53 immunohistochemical expression. In a study with 332 T1-2 N-negative breast cancers (Kühling et al. 2003, Rudolph et al. 2003), high cyclin B1 expression was associated with relapse-free and overall survival in univariate analysis, but was not an independent prognostic factor in a multivariate analysis that included Ki67 as a covariate (Kühling et al. 2003). When 273 tumors treated with surgery and postoperative radiation only were analyzed, cyclin B1 was an independent predictor of poor overall survival among premenopausal but not postmenopausal patients (Rudolph et al. 2003). Tumors with high cyclin B1 expression were more often of high grade, ER- and PR-negative, and also had high Ki67, cyclin A, and cyclin E expression. In a small study with 56 invasive stage I-II cancers, cyclin B1 was not associated with prognosis (Peters et al. 2004). A recent study with 109 breast cancers showed an association between only nuclear cyclin B1 expression and prognosis (Suzuki et al. 2007).

2.3.5 Cyclin D1

Cyclin D1-CDK4/6 complex is a critical regulator of G1/S transition through pRb phosphorylation and titration of p21 and p27 levels, as described earlier (Sherr 1996). The major function of cyclin D1 is to integrate stimuli from extracellular mitogenic factors, such as tyrosine kinases and hormones, with cell cycle progression during the G1 phase (Kato et al. 1993). After mitogenic activation, cyclin D1/CDK4/6 complex begins pRb phosphorylation, but also sequesters CDK inhibitors, e.g. p27 and p21, thus relieving cyclin E/CDK2 complex of its inhibitory control. Estrogen directly targets cyclin D1, and cyclin D1/CDK 4/6 complex level rises shortly after estrogen stimulation (Doisneau-Sixou et al. 2003). pRb is an important substrate of cyclin D1/CDK4/6 complex; in cells lacking pRb, cyclin D1 is not required for cell cycle progression. Cyclin D1 seems to have CDK-independent functions, too, which contribute to its oncogenic actions (Arnold & Papanikolaou 2005). Cyclin D1 may activate estrogen receptor without estrogen, and thus, drive all the mitogenic effects of estrogen on breast cells (Neuman et al. 1997, Lamb et al. 2000).

2.3.6 Cyclin D1 in breast cancer

Cyclin D1 is overexpressed in about 50% of breast cancers, partly due to CCND1 gene amplification and partly due to translational or posttranslational deregulation (Gillett et al. 1994). CCND1 gene amplification occurs in about 15-20% of breast cancers. Cyclin D1 overexpression has also been detected in ductal hyperplasia and ductal carcinoma in situ (DCIS), indicating that it may have a role in the evolution of early breast cancer (Wang et al. 1994). Breast cancers with high cyclin D1 expression are more frequently hormone receptor-positive than -negative. The association of cyclin D1 with tumor grade and proliferation is not fully understood; associations between high cyclin D1 levels and low-grade tumor and low proliferation rate have been reported (van Dienst et al. 1997, Han et al. 2003, Hwang et al. 2003, Jirström et al. 2005), but not consistently in all studies (Michalides et al. 1996, Umekita et al. 2002, Reis-Filho et al. 2006). The relationship between cyclin D1 and HER2 overexpression also remains unclear. One study on 113 primary breast cancers and breast cancer cell lines suggested that proliferation is increased in hormone receptor-positive tumors with high cyclin D1 (Loden et al. 2002). Cyclin D1

does not seem to have an independent prognostic role in breast cancer; there are several studies that have been unable to show any association and a few have even shown an association of high cyclin D1 with better prognosis (Michalides et al. 1996, van Dienst et al. 1997, Gillett et al. 1998, Umekita et al. 2002, Reis-Filho et al. 2006, Elsheikh et al. 2007). Instead of being a prognostic factor, cyclin D1 has been suggested to mediate tamoxifen resistance (Stendahl et al. 2004, Ahnström et al. 2005, Jirström et al. 2005).

2.3.7 Cyclin E

Cyclin E expression rises and cyclin E/CDK2 complex becomes released from CDK inhibitors p27, p21, and others at the G1/S transition as a result of activity of cyclin D1/CDK4/6. Activated E2F can induce cyclin E as well and creates a positive feedback loop that contributes to the irreversibility of the G1/S transition. Cyclin E/CDK2 carries on the inactivation of pRb later in G1 and enables further upregulation of genes needed for DNA replication (Sherr 1996). Cyclin E also facilitates the first events of the S phase. Cyclin E/CDK2 complex is capable of phosphorylating substrates besides pRb that mediate its proliferative actions, and cyclin E can accelerate entry into S phase independently of pRb (Ohtsubo et al. 1995). One of these substrates is the p27 inhibitor, and cyclin E facilitates its own activation by phosphorylating p27. In normal cells, cyclin E becomes quickly degraded by multiple mechanisms, and during early G1 and G2/M phases its level and activity are low.

Cyclin E overexpression can cause chromosomal instability, potentially by disturbing proper chromosome duplication and segregation (Spruck et al. 1999). Cyclin E overexpression is common in cancer. Amplification of its coding gene CCNE is unusual; instead, posttranslational deregulation seems to be the mechanism leading to high cyclin E expression (Keyomarsi et al. 1995). Further splicing of the CCNE transcript as well as posttranslational proteolytic cleavage of the cyclin E protein lead to the formation of low molecular weight (LMW) isoforms of cyclin E found in tumor tissues (Porter & Keyomarsi 2000, Porter et al. 2001).

2.3.8 Cyclin E in breast cancer

Cyclin E expression has been shown to be often high in breast cancers with an aggressive tumor phenotype. High cyclin E expression correlates with hormone receptor negativity, high tumor grade, and large tumor size (Nielsen et al. 1996, Donnellan et al. 2001, Keyomarsi et al. 2002, Han et al. 2003, Rudolph et al. 2003, Lindahl et al. 2004). Furthermore, correlations between high cyclin E and high Ki67 count and younger age at diagnosis have been reported (Kühling et al. 2003, Lindahl et al. 2004, Berglund et al. 2008). One study found that high cyclin E was more common among HER2-positive tumors (Potemski et al. 2006). Medullary histology has been described as more common among tumors with high than low cyclin E expression, suggesting cyclin E's ability to modulate the infiltrative behavior of the tumor (Berglund et al. 2005). Tumors with high cyclin E have also been shown to have a specific p53 mutation spectrum, with mainly insertions and deletions and undetectable p53 expression (Lindahl et al. 2004). Most studies evaluating the prognostic role of cyclin E in breast cancer have revealed an association with poor prognosis (Nielsen et al. 1996, Keyomarsi et al. 2002, Han et al. 2003, Kühling et al. 2003, Rudolph et al. 2003, Lindahl et al. 2004, Chappuis et al. 2005, Spruck et al. 2006). In some of these studies, an independent effect on poor prognosis in multivariate analysis could not be found. Some studies have suggested that the prognostic effect can only be seen when the LMW forms of cyclin E are measured. High cyclin E has also been speculated to predict endocrine therapy failure (Span et al. 2003, Akli et al. 2004, Desmedt et al. 2006). One study consisting only of patients treated with adjuvant chemotherapy showed no adverse prognostic effect (Porter et al. 2006). A meta-analysis with 2534 patients, however, revealed that high cyclin E was associated with poor relapse-free survival (RR 1.72, 95% CI 0.95-3.10) and with poor overall or breast cancer-specific survival (RR 2.86, 95% CI 1.85-4.41) also in multivariate analysis (Wang & Shao 2006). Thus, high cyclin E may be an independent predictor of poor breast cancer survival.

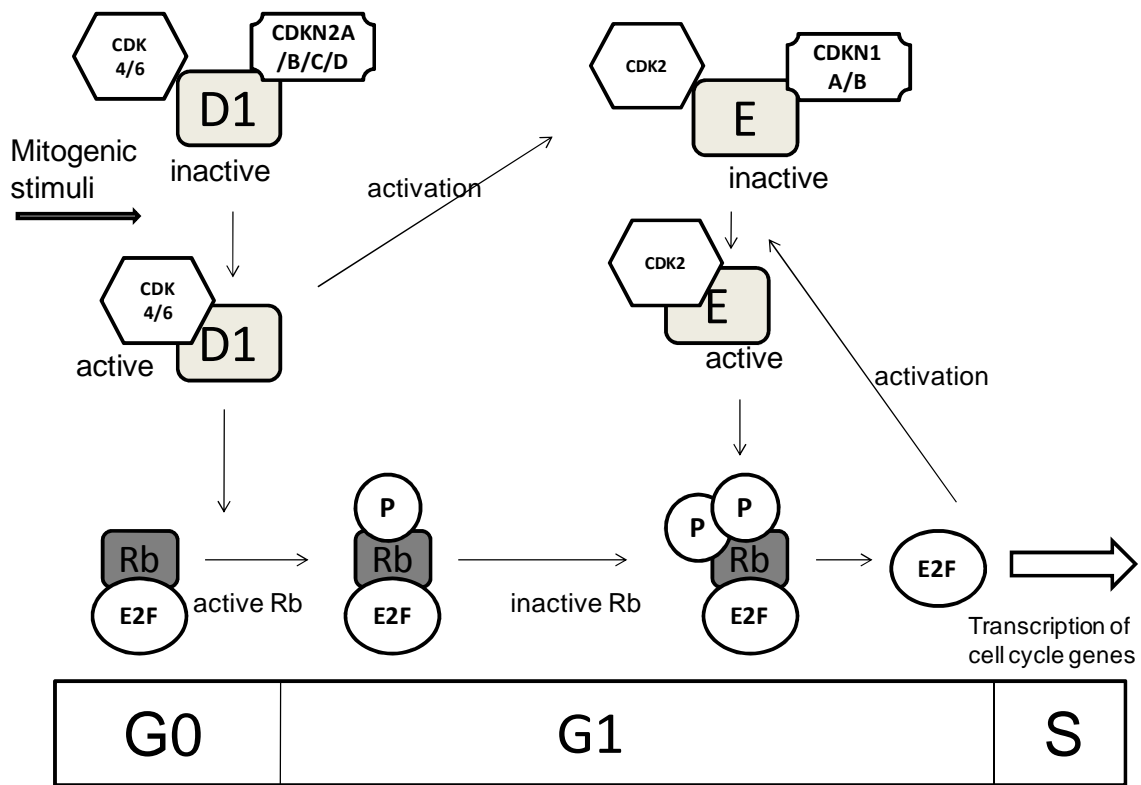


Figure 2 *Simplified model of cell cycle G1 to S transition (adapted from Sherr & Roberts 1999).*

2.3.9 Proliferation antigen Ki67

Ki67 is a nuclear protein that is absent in quiescent cells, but universally expressed in proliferating tissues (van Dierendonck et al. 1989, Gerdes et al. 1991), and thus, can be used as a marker of cell proliferation. Ki67 level in cells rises during G1 and S phases and reaches its maximum during mitosis. Ki67 seems to have a critical role in cell division, but little is known about its exact function (Heidebrecht et al. 1996). While the prognostic role of Ki67 in early breast cancer has been unclear, a recent meta-analysis suggests a significant association with poor disease-free and overall survival in univariate analysis (de Azambuja et al. 2007). Because multivariate prognostic analyses were not conducted, the independent prognostic value of Ki67 cannot be determined. Furthermore, uncertainty remains about the optimal cut-off values of Ki67 (Urruticoechea et al. 2005). A study investigating the optimal cut-off values of cyclin A and Ki67 in prognostic evaluation suggested the optimal cut-off value for identifying tumors with poor prognosis for Ki67 to be around 15% (Ahlin et al. 2007).

2.4 Tissue microarray (TMA) technique

In the tissue microarray (TMA) technique (Kononen et al. 1998), small cylinders (diameter 0.6 μm) from hundreds of separate tumors are punched and brought into a single recipient TMA block. Sections of these blocks can then be used in tumor marker analyses on a DNA, RNA, and protein level. This technique allows simultaneous staining and analysis of a large number of tumors, enabling rapid, large-scale investigations of new tumor markers. Since the TMA technique takes only small cylindrical specimens from the donor blocks, these donor blocks remain virtually undamaged and can be used for several studies.

As only a small punch (0.6 μm) from each tumor is analyzed on TMA, a question has arisen of the representativeness of this minute tissue punch and the possible effect of tumor heterogeneity on results. Several studies have shown that although an individual result on TMA and on a corresponding large-section slide may differ, the correlations between histopathological features and prognostic implications are similar when large tumor materials are investigated (Kononen et al. 1998, Camp et al. 2000, Gillett et al. 2000, Nocito et al. 2001, Torhorst et al. 2001). ER, PR, and HER2 expression has been examined on breast cancer TMAs, and the results have been reliable (Kononen et al. 1998, Camp et al. 2000, Gillett et al. 2000, Torhorst et al. 2001). Expression of cell cycle regulators cyclin D1 and E also show similar results on TMA and on traditional large-section slides (Richter et al. 2000, Han et al. 2003, Hedberg et al. 2003, Jirström et al. 2003, Schraml et al. 2003, Stendahl et al. 2004). It has, however, been unclear how representative TMA is when studying markers with heterogenic expression in different parts of a tumor (Gillett et al. 2000).

3 AIMS OF THE STUDY

This study aimed to bring new insights into the biological roles and clinical implications of cell cycle regulator protein cyclins in breast cancer. Specific aims were to evaluate:

1. the reliability and reproducibility of cyclin A immunohistochemical assessment on TMA compared with traditional histological slides
2. cyclin D1, E, and B1 expression in familial breast cancer, focusing on differences in expression between tumors of sporadic, familial non-BRCA1/2, BRCA1-, and BRCA2-positive breast cancer patients
3. the role of cyclin D1 gene (CCND1) G870A polymorphism in breast cancer predisposition alone and in combination with estrogen metabolism enzyme COMT Met108/158Val polymorphism
4. the associations of cyclin D1, E, and B1 expression with breast cancer phenotype, focusing on cell cycle regulation and proliferation and with breast cancer prognosis

4 PATIENTS AND METHODS

4.1 Patients

4.1.1 Breast cancer TMAs (I, II, IV, V)

The breast cancer TMAs consist of 1348 invasive breast tumors and constitute the material in Studies II, IV, and V. Of these tumors 884 are from unselected patients of the Department of Oncology, Helsinki University Central Hospital, seen in 1997, 1998, and 2000 (79% of all consecutive, newly diagnosed breast cancer cases during the collection periods). The unselected series have been described in detail by Syrjäkoski et al. (2000) and Kilpivaara et al. (2005). Of the unselected tumors, 439 are from sporadic and 445 from patients with one first-degree or two or more first- or second-degree relatives with breast or ovarian cancer. An additional 464 tumors from familial breast cancer patients were located by a systematic screening at the Department of Oncology, Helsinki University Central Hospital, or were ascertained through genetic counseling at the Department of Clinical Genetics (described by Eerola et al. 2000). Of all familial patients' tumors, 453 are from patients with a strong positive family history (at least three first- or second-degree relatives with breast or ovarian cancer, including the proband), 341 from patients with two affected first-degree relatives (including the proband), 56 from BRCA1 mutation carriers, and 59 from BRCA2 mutation carriers. The BRCA1 and BRCA2 mutation screening of familial tumors has been described earlier in Vehmanen et al. (1997) and Vahteristo et al. (2001).

In Study I, 200 tumors from the unselected series collected between 1997 and 1998 were randomly selected and cyclin A expression of these tumors was examined both on TMAs and on traditional large section histological slides.

4.1.2 Characterization of tumors (I, II, IV, V)

All cancer diagnoses were confirmed through the Finnish Cancer Registry. Noninvasive cancers were excluded. Information on tumor histology, grade, size, nodal status, distant metastases, and ER and PR status was obtained from pathology reports. The tumors were

considered positive for ER and PR if 10% or more of the cancer cells were stained. An expert breast cancer pathologist re-reviewed all tumors for tumor histology and grade (Eerola et al. 2005). Grading was performed according to Scarff-Bloom-Richardson (1957), modified by Elston and Ellis (1991). HER2 protein expression on TMAs was analyzed by immunohistochemical staining and gene amplification with chromogenic in situ hybridization (CISH), and p53 protein expression by immunohistochemical expression. The methods for HER2 analysis have been described in detail by Tanner et al. (2000) and Lassus et al. (2004) and for p53 immunohistochemistry by Tammiska et al. (2005).

4.1.3 Follow-up data (IV, V)

Information on adjuvant treatment and distant metastases during the follow-up was collected from patient records. Information on death due to breast cancer or another reason came from the Finnish Cancer Registry. Survival was analyzed as metastasis-free survival (MFS); the time from the date of primary surgery to the date of radiological confirmed distant metastases, and as overall survival (OS); the time from the date of primary surgery to the date of death due to breast cancer. A total of 797 patients were accepted to survival analysis in Studies IV and V, including the unselected series and the familial patients ascertained within 6 months after diagnosis. The median follow-up time of these patients was 93 (2-516) months. The patients were treated according to standard guidelines at that time regarding adjuvant chemotherapy, radiotherapy, and endocrine treatment. Of these 797 patients, 796 (99.9%) underwent surgery, 691 (87%) adjuvant radiotherapy, 323 (41%) adjuvant chemotherapy, and 359 (45%) adjuvant endocrine treatment. Of the patients who received adjuvant chemotherapy, 163 (50%) were treated with CMF (cyclophosphamide-methotrexate-5-fluorouracil), 102 (32%) with CEF (cyclophosphamide-epirubicin-5-fluorouracil), and 58 (18%) with some other chemotherapy regimen. Of all patients in the survival analysis, 127 (16%) relapsed with distant metastases during the follow-up time, 91 (11%) of whom then died from breast cancer.

4.1.4 Breast cancer patients in Study III

All Finnish breast cancer cases (n=728) were included in the unselected breast cancer series collected between 1997-1998 and 2000. Of these cases, 73% (n=534) were sporadic, with no family history of breast or ovarian cancer, and 27% (n=194) had at least one first-degree relative with breast or ovarian cancer. The age range of these cases was 22-69 years, the average age being 53.2 ± 9.34 years.

The breast cancer cases from Ontario, Canada (n=1228), were identified through the Ontario Cancer Registry and recruited into the Ontario Familial Breast Cancer Registry (OFBCR). They were diagnosed with pathologically confirmed breast cancer between 1996 and 1998. Of these cases, 73% represent women at increased risk of genetically related breast cancer (Ashkenazi Jewish background, diagnosed before age 36 years, previous ovarian or breast cancer diagnosis, one or more first- or two or more second-degree relatives with breast or ovarian cancer, one or more second- or third-degree relatives with either breast cancer diagnosed before age 36 years, ovarian cancer diagnosed before age 61 years, multiple breast or breast and ovarian primaries, or male breast cancer, or three or more first-degree relatives with any combination of breast, ovarian, colon, prostate, or pancreatic cancer or sarcoma, with at least one diagnosis before age 51 years) and 35.6% had one or more first-degree relatives with breast or ovarian cancers. The age range of these cases was 25-69 years, the average age being 48.8 ± 9.26 years.

4.1.5 Population controls (III)

The population controls in the Study III were collected from Finland and Ontario, Canada. The Finland population controls (n=687) comprised healthy females collected from the same geographical region as the cases. The age range of the Finland controls was 21-65 years, the average age being 47.1 ± 10.12 years. The Ontario population controls (n=719) were recruited from the OFBCR by calling randomly selected residential telephone numbers from across the province of Ontario and were frequency-matched to all female OFBCR cases by 5-year age group. The age range of the Ontario population controls was 23-69 years, the average age being 49.1 ± 9.55 years.

4.2 Methods

4.2.1 TMA construction (I, II, IV, V)

Paraffin blocks from the patients' primary tumors were collected. Hematoxylin and eosin sections were reviewed, and the most representative tumor areas were selected. These areas from each tumor were punched and placed on recipient paraffin blocks to produce TMAs consisting of four cores with diameter 0.6 μ m (two cores from BRCA-positive tumors) for each tumor. Then 3- to 4- μ m-thick sections were cut from array blocks and transferred to glass slides (Eerola et al. 2005).

4.2.2 Immunohistochemistry (I, II, IV, V)

Deparaffinization of the TMA samples was performed using xylene. The slides were rehydrated through graded alcohols to water. Cyclin A immunostaining was performed manually. Antigen retrieval was done using a pressure cooker for 5 min in 0.01 M citrate buffer, pH6.0. Primary antibody (mouse monoclonal, Novocastra Laboratories) was diluted 1:300 and incubated overnight. Staining was done using the avidin biotin peroxidase complex and amino-ethyl-carbazole (AEC) procedures (Wood & Warnke 1981). Cyclin B1, D1, and E and Ki67 immunostainings were performed in an automated immunostainer (Ventana Medical Systems Inc.) using a diaminobenzidine (DAB) kit (Ventana). Antigen retrieval was done using an iView-kit. Cyclin E (BD Pharmingen) and cyclin D1 (Novocastra) were diluted 1:20, and cyclin B1 (Novocastra) 1:40. Ki67 (Mib-1, Dako cytometry) was diluted 1:100.

The formalin-fixed paraffin-embedded tissue material from the blocks of the 200 tumors in Study I was cut into 3- to 4- μ m-thick sections and deparaffinized. The immunostaining for cyclin A was performed similarly to the immunostaining of TMAs.

4.2.3 Evaluation of immunoreactivity scores (I, II, IV, V)

Immunostainings on TMA slides were analyzed by at least one and sometimes two investigators. All scoring was done under the supervision of an experienced breast cancer pathologist. The cyclin A immunostaining of the 200 tumors in Study I was analyzed on

both TMAs and traditional large-section histological slides by two investigators. For all immunostainings, the number of positive breast cancer cells was counted in one high-power field (40X objective) in each of the four tissue cores on TMA. Only unequivocal nuclear staining was accepted as a positive reaction for cyclin A, cyclin D1, cyclin E, and Ki67. For cyclin B1, both nuclear and cytoplasmic reactions were accepted as positive. A minimum of 200 cells was counted in each tumor, except for cyclin B1 immunostaining, where a minimum of 500 cells was counted. The result was the proportion of all positive cells of the total number of breast cancer cells counted from the four biopsies. In Study I, the maximal and the average values of the four cores were used in analyses, and in the large sections three randomly selected and one “hot-spot” high-power field were evaluated.

Most of the analyses were performed using cyclin and Ki67 results as continuous variables, but to better demonstrate some of the histopathological associations and prognostic effects, the results were dichotomized at mean values. In Study I, cyclin A was dichotomized at a cut-off value of 10% (Poikonen et al. 2005), and in Study V, cyclin B1 was dichotomized at 5.6% (corresponding to the 7th decile).

4.2.4 DNA extraction (III)

A standard phenol-chloroform method was used to extract genomic DNA from peripheral blood leukocytes of the breast cancer patients and controls.

4.2.5 Molecular genotyping (III)

The genotyping of the Finnish breast cancer and population control DNA specimens for COMT and CCND1 polymorphisms was done using Amplifluor fluorescent genotyping (K-Biosciences, Cambridge, UK) (Tommiska et al. 2005). The genotyping of the DNA samples from the Canadian breast cancer cases and controls was performed using TaqMan 5' nuclease assay with the ABI PRISM 7900 HT Sequence Detection System (version 2.0) (Onay et al. 2006). A randomly selected 10% portion of the total study population was re-genotyped to evaluate the reliability of the results.

4.2.6 Statistical methods

Statistical software package SPSS version 13.0 for Windows (SPSS, Chicago, IL, USA) or SISA (<http://home.clara.net/sisa/>) was used for all analyses. Two-sided P-value of 0.05 was considered to indicate statistical significance, unless otherwise stated.

The reproducibility of the two readers' results and the results achieved by two methods was evaluated with Kappa values. Correlations were assessed with either Mann-Whitney U-test (dichotomized variables) or Spearman's rho correlation test (continuous variables). Logistic regression analysis (stepwise backwards logistic regression, 99%) was used in multivariate analysis of associations. Survival was analyzed with Cox regression analysis in univariate and multivariate models. Kaplan-Meier survival curves were calculated. To determine the optimal cut-off value for separation of tumors with good versus poor prognosis, the material was divided into 10 deciles. The cut-off values corresponding to each decile limit were used to split the material into two parts. For each cut-off, the relative risk (RR) for survival with 95% confidence interval (CI) was calculated using the Cox regression analysis.

The associations of SNPs with controls and cases were measured by odds ratio (OR) and its corresponding 95% CI. The Trend Analysis Program (PEPI computer software package, Sagebrush Press, Salt Lake City state, USA) was applied to detect trends from SNP interactions.

4.3 Ethics

All studies were carried out with the informed consent of patients and were approved by the Ethics committee of the Helsinki University Central Hospital and the Ministry of Social Affairs and Health in Finland.

5 RESULTS

5.1 Cyclin A assessment on TMA (I)

Because cyclin A expression is heterogeneous throughout the tumor, we evaluated the reliability of cyclin A assessment on TMAs, where only small punches from each tumor are analyzed. Both maximum and average cyclin A counts were studied. Of 200 tumors investigated, the results of 14 tumors (7%) were missing on TMA, due to either loss of all punches during the staining or lack of tumor in the arrayed samples. The result of only one tumor was missing on large sections, due to unsuccessful staining. The median cyclin A count was 3.7% (range 0–34.4%) on TMA average values, 5.8% (0–52.9%) on TMA maximum values, 4.3% (0–32.1%) on large-section average values, and 9.0% (0–39.1%) on large-section maximum values.

5.1.1 Agreement of two readers' results

The two readers' results were concordant in 173 cases (96%) on TMA average values, in 167 cases (92%) on TMA maximum values, in 174 cases (87%) on large-section average values, and in 180 cases (90%) on large-section maximum values. The mean difference between the two readers' scoring and 95% limits of agreement were 0.1% (-4.8% – +5.1%) for TMA average values, 0.4% (-8.0% – +8.8%) for TMA maximum values, 0.4% (-4.4% – +5.1%) for large-section average values, and 1.0% (-7.5% – +9.5%) for large-section maximum values. The kappa values evaluating the reproducibility of two readers' results were 0.87 for TMA average value, 0.83 for TMA maximum value, 0.71 for large-section average value, and 0.80 for large-section maximum value. The mean number of nuclei counted (the mean of two readers' results) was 661 in concordant tumors versus 420 in discrepant tumors ($P=0.002$) for array average values, 671 in concordant versus 410 in discrepant tumors ($P<0.0005$) for array maximum values, 767 in concordant versus 465 in discrepant tumors ($P<0.0005$) for large-section average values, and 759 in concordant versus 518 in discrepant tumors for large-section maximum values ($P<0.0005$).

5.1.2 Agreement of cyclin A results on TMA and large sections

TMA and large-section cyclin A results were compared using the median values of the two readers' results. Of average values 171 (92%) were concordant, and of maximum values 152 (82%). The mean difference between the TMA and large-section scoring and 95% limits of agreement were 0.4% (-6.9% – +7.6%) for average values and 2.0% (-8.7% – +12.6%) for maximum values. The kappa values were 0.75 for average values and 0.62 for maximum values. The mean number of nuclei counted was 683 in concordant tumors and 308 in discrepant tumors for average values ($P<0.0005$). For maximum values, the mean number of cells counted in concordant tumors was 661 and in discrepant tumors 612 ($P=0.33$). The associations of cyclin A with other histopathological factors and survival were similar in TMA average and maximum values as well as in large-section average and maximum values. High cyclin A expression was associated with ER negativity ($P<0.0005$), PR negativity ($P<0.0005$), and high grade ($P<0.0005$). High cyclin A was associated with poor MFS, but did not have a significant effect on OS.

Table 4 Cyclin A correlations with tumor characteristics.

	Spearman's correlation coefficient	P-value (Mann-Whitney-U test)
<i>Tumor grade</i>		
TMA average	0.529	<0.0005
TMA maximum	0.537	<0.0005
large-section average	0.555	<0.0005
large-section maximum	0.523	<0.0005
<i>Nodal status</i>		
TMA average	-0.078	0.38
TMA maximum	-0.065	0.45
large-section average	-0.046	0.58
large-section maximum	-0.023	0.80
<i>ER</i>		
TMA average	-0.417	<0.0005
TMA maximum	-0.414	<0.0005
large-section average	-0.453	<0.0005
large-section maximum	-0.459	<0.0005
<i>PR</i>		
TMA average	-0.427	<0.0005
TMA maximum	-0.427	<0.0005
large-section average	-0.453	<0.0005
large-section maximum	-0.459	<0.0005

5.2 CCND1 G870A and COMT Met108/158Val polymorphisms (III)

The series of 728 breast cancer cases from Finland and 1223 from Ontario, Canada, and 687 population controls from Finland and 719 from Ontario were screened for CCND1 G870A and COMT Met108/158Val polymorphisms. The frequency of COMT high enzymatic activity allele (Val) was 0.45 in Finnish and 0.47 in Canadian control populations, and the frequency of CCND1 high enzymatic activity allele (A) was 0.46 in both control populations. No deviation from the Hardy-Weinberg equilibrium was detected.

5.2.1 Association of COMT Met108/158Val polymorphism with breast cancer risk

The heterozygous medium enzymatic activity COMT MetVal genotype and the homozygous high enzymatic activity COMT ValVal genotype were associated with increased breast cancer risk in Canadian cases (OR 1.3, 95% CI 1.07-1.68 and OR 1.4, 95% CI 1.07-1.81, respectively). Among Finnish cases, however, no significant association or increased trend for breast cancer risk with COMT MetVal or ValVal genotypes (OR 1.0, 95% CI 0.73-1.39) was found.

A meta-analysis of thirteen studies with genotype data from 6809 cases and 6190 controls was carried out to assess the association of COMT Met108/158Val polymorphism with breast cancer risk. Overall fixed-effects for pooled OR were slightly increased, but not significant for MetMet versus ValVal genotype (OR 1.08, 95% CI 0.93–1.24, $P=0.32$). The genotype distribution of the control populations in two studies deviated from the Hardy-Weinberg equilibrium. The meta-analysis was repeated after removing these two studies, after which a significant association with breast cancer risk for MetMet vs. ValVal genotype (OR 1.14, 95% CI 1.03-1.26, $P=0.01$) with no heterogeneity (MetMet vs. ValVal: P heterogeneity=0.58; $I^2=0\%$) was detected.

5.2.2 Association of CCND1 G870A polymorphism with breast cancer risk

The high enzymatic activity CCND1 AA genotype was associated with increased breast cancer risk in both Canadian (OR 1.3, 95% CI 1.00-1.69) and Finnish samples (OR 1.4, 95% CI 1.01-1.84).

5.2.3 Characteristics of tumors from patients with CCND1 AA genotype

Characteristics of the tumors from 728 Finnish cases were studied. Tumors of patients with AA, AG, or GG genotypes were very similar, and the AA genotype had no significant association with tumor TNM status, grade, histology, hormone receptor status, HER2, or p53 or Ki67 count. Nor did the CCND1 G870A genotype and cyclin D1 expression correlate.

5.2.4 Combined effect of CCND1 G870A and COMT Met108/158Val polymorphisms

The association of the combined CCND1 and COMT genotypes with breast cancer predisposition was also evaluated. The low enzymatic activity genotype combination of CCND1 and COMT (CCND1 GG/COMT MetMet) was taken as a reference and compared with the medium- (heterozygote combinations) and high-activity (CCND1 AA/COMT MetVal and CCND1 AA/COMT ValVal) combinations. In the Canadian series, the heterozygote and the high-activity genotype combinations showed significant associations with increased breast cancer risk (OR 1.66, 95% CI 1.18-2.33 for medium- and OR 2.22, 95% CI 1.49-3.28 for high-activity combination). In the Finnish series, the high-activity genotype combinations were also significantly associated with increased breast cancer risk (OR 1.73, 95% CI 1.08-2.78). The medium-activity combinations in the Finnish sample showed a trend of increased breast cancer risk (OR 1.21, 95% CI 0.81-1.83), but did not reach statistical significance. The COMT and CCND1 genotype interactions were also investigated in subgroups defined by age, familial status, and ER status; the associations were, however, similar to those in the overall analysis.

The Canadian study was designed to reach 80% power in detecting an OR of about 2.1 for the interaction, assuming a recessive model for CCND1 and a dominant model for COMT

using a two-sided test. Although the Finnish study included less cases and controls, it achieved almost the same power, assuming the direction of the effect is known and a one-sided test can be used; both assumptions are justified in a replication study.

5.3 Cyclin D1 and E expression (II, IV)

The TMAs with 1348 invasive breast cancers were stained with cyclin D1 and E antibodies to investigate the associations of these markers with other tumor characteristics, familial background, and prognosis. The cyclin D1 result was obtained from 1187 tumors (88.1%) and the cyclin E results from 1180 tumors (87.5%). In the remaining cases, the biopsy did not contain enough tumor cells to be evaluated or the staining was unsuccessful. The median cyclin D1 count was 9.0% (range 0-81%) and the median cyclin E count 6.5% (range 0-68%). The tumors with expression above the mean expression of all tumors (6.8% for cyclin E and 9.1% for cyclin E) were considered high-expression tumors (positive) and those below the mean expression low-expression tumors (negative).

5.3.1 Cyclin D1 and E expression among tumors of BRCA1 carriers

Of BRCA1 tumors, 88% had high cyclin E expression and 84% had low cyclin D1 expression. High cyclin E and low cyclin D1 count were significantly more common among BRCA1 than among familial non-BRCA1/2 ($P<0.0005$) or sporadic tumors ($P<0.0005$). Other factors differentiating BRCA1 tumors from familial non-BRCA1/2 tumors or sporadic tumors in univariate analysis were high tumor grade, younger age at diagnosis, and higher frequency of ER-, PR-, and HER2-negative, p53- and Ki67-positive tumors, and tumors with medullar histology. BRCA1 tumors also more frequently had negative nodal status than sporadic tumors. A multivariate model taking into account all factors significant in univariate analysis was constructed. The independent differentiating factors between BRCA1 and familial non-BRCA1/2 tumors were high cyclin E and low cyclin D1 expression, HER2 and ER negativity, and younger age at diagnosis. The factors distinguishing BRCA1 from sporadic tumors in multivariate analysis were ER and HER2 negativity and younger age at diagnosis. High cyclin E and low cyclin D1 expression were, however, not significant in multivariate analysis.

Table 5 *Logistic regression analysis (first and final step) of BRCA1-associated breast cancer features in comparison with familial non-BRCA1/2 tumors and sporadic tumors.*

A. familial non-BRCA1/2 tumors			B. sporadic tumors		
Feature	OR (95% CI)	P-value	Feature	OR (95% CI)	P-value
<i>First step:</i>			<i>First step:</i>		
HER2 neg	2.17 E+08*	0.997*	HER2 neg	2.15 E+08*	0.997*
Cyclin E pos	5.36 (1.47-19.61)	0.01	Cyclin E pos	2.33 (0.62-8.71)	0.21
Cyclin D1 neg	4.63 (1.35-15.85)	0.02	Cyclin D1 neg	2.83 (0.78-10.31)	0.11
ER neg	3.14 (0.66-14.96)	0.15	ER neg	6.85 (1.19-39.26)	0.03
Age <50 years	3.28 (1.25-8.63)	0.02	Age <50 years	4.53 (1.64-12.47)	0.003
P53 pos	2.45 (0.82-7.34)	0.11	P53 pos	1.22 (0.43-3.50)	0.71
PR neg	1.71 (0.35-8.32)	0.51	PR neg	2.20 (0.45-10.70)	0.33
Grade 3	0.73 (0.21-2.57)	0.62	Grade 3	1.97 (0.52-7.54)	0.32
			Nodal status neg	1.40 (0.54-3.62)	0.49
<i>Final step after stepwise regression:</i>			<i>Final step after stepwise regression:</i>		
HER2	2.19 E+08*	0.997*	HER2 neg	2.55 E+08*	0.997*
Cyclin E pos	5.58 (1.69-13.67)	0.01	ER neg	15.33 (6.06-38.75)	<0.0005
ER neg	3.81 (1.35-10.76)	0.01	Age <50 years	4.40 (1.76-11.00)	0.002
Cyclin D1 neg	4.36 (1.33-14.22)	0.02			
Age <50 years	2.99 (1.17-7.65)	0.02			

* All BRCA1 and BRCA2 tumors with HER2 result in this study were HER2 negative.

Prevalence of combined phenotypes defined by the independent differentiating factors (high cyclin E expression, low cyclin D1 expression, younger age at diagnosis, ER, and HER2 negativity) were further compared between mutation-positive and familial mutation-negative tumors to evaluate predictive value of these markers for BRCA1 mutation. Including both high cyclin E and low cyclin D1 expression increased the OR of BRCA1 mutation to 27.82 and high cyclin E to 28.85, as compared with an OR of 19.12 for traditional markers ER, HER2, and age at diagnosis alone.

Table 6 *Prevalence of combined immunotypes by ER, HER2, age at diagnosis, and cyclin E and D1 expression in BRCA1 and BRCA1 mutation-negative (BRCA2 and non-BRCA1/2) familial tumors.*

ER	HER2	AGE AT DIAGNOSIS	CYCLIN D1	CYCLIN E	BRCA1 (%)	FAMILIAL BRCA1 NEG (%)	OR (95% CI)
neg					22 (75.9%)	84 (23.7%)	10.10 (4.17-24.48)
neg	neg				22 (75.9%)	68 (19.2%)	13.22 (5.42-32.21)
neg	neg	<50 years			19 (65.5%)	32 (9.0%)	19.12 (8.19-44.62)
neg	neg	<50 years	neg		17 (58.6%)	23 (6.5%)	20.39 (8.70-47.76)
neg	neg	<50 years		pos	18 (62.1%)	19 (5.4%)	28.85 (11.95-69.63)
neg	neg	<50 years	neg	pos	16 (55.2%)	15 (4.2%)	27.82 (11.35-68.15)

5.3.2 Cyclin D1 and E expression among tumors of BRCA2 carriers

High cyclin E expression was seen in 58% and low cyclin D1 in 69% of BRCA2 tumors. High cyclin E was significantly more common among BRCA2 than familial non-

BRCA1/2 (P=0.01) and low cyclin D1 significantly more common among BRCA2 than familial non-BRCA1/2 (P=0.002) or sporadic (P=0.009) tumors. Other factors differentiating BRCA2 tumors from familial non-BRCA1/2 and sporadic tumors in univariate analysis were negative ER, PR, and HER2 status and younger age at diagnosis. BRCA2 tumors were also more frequently N-, M-, and Ki67-positive than familial non-BRCA1/2 tumors and more often of lobular histology than sporadic tumors. In multivariate analysis, the independent factors differentiating BRCA2 tumors from familial non-BRCA1/2 tumors were high cyclin E and low cyclin D1 expression, HER2 negativity, younger age at diagnosis, and negative nodal status. In comparison with sporadic tumors, only younger age at diagnosis and negative PR and HER2 status were independent differentiating factors.

Table 7 *Logistic regression analysis (first and final step) of BRCA2-associated breast cancer features in comparison with familial non-BRCA1/2 tumors and sporadic tumors.*

A. familial non-BRCA1/2 tumors			B. sporadic tumors		
Feature	OR (95% CI)	P-value	Feature	OR (95% CI)	P-value
<i>First step:</i>			<i>First step:</i>		
Nodal status neg	0.39 (0.16-0.95)	0.04	ER neg	0.93 (0.28-3.13)	0.90
M neg	0.36 (0.05-2.36)	0.28	PR neg	2.09 (0.78-5.64)	0.15
PR neg	1.41 (0.54-3.69)	0.48	HER2 neg	2.29 E+10*	0.997*
HER2 neg	2.13 E+08*	0.998*	Age <50 years	5.35 (2.38-12.02)	<0.0005
Age <50 years	3.83 (1.48-9.91)	0.01	High cyclin E	1.12 (0.27-4.68)	0.88
High cyclin E	4.04 (1.49-10.93)	0.006	Low cyclin D1	1.34 (0.33-5.49)	0.69
Low cyclin D1	2.16 (0.83-5.61)	0.11			
<i>Final step after stepwise regression:</i>			<i>Final step after stepwise regression:</i>		
HER2 neg	2.13 E+08*	0.998*	HER2 neg	2.29 E+10*	0.997*
Age <50 years	3.75 (1.50-9.39)	0.01	PR neg	2.63 (1.23-5.63)	0.01
High cyclin E	3.69 (1.41-9.20)	0.007	Age <50 years	6.01 (2.72-13.29)	<0.0005
Low cyclin D1	2.26 (0.93-5.54)	0.07			
Nodal status neg	0.38 (0.16-0.89)	0.03			

* All BRCA1 and BRCA2 tumors with HER2 result in this study were HER2 negative.

5.3.3 Cyclin D1 and E expression among tumors of familial non-BRCA1/2 patients

Of familial non-BRCA1/2 tumors, 38% had high cyclin E and 45% low cyclin D1 expression. As mentioned above, in univariate analysis, the frequency of tumors with high cyclin E expression was significantly lower than among BRCA1, BRCA2, or sporadic tumors (P<0.0005). The number of tumors with low cyclin D1 was significantly less than among BRCA1 or BRCA2 tumors, and somewhat but not significantly less than among

sporadic tumors (P=0.05). In univariate analysis, the other features differentiating familial non-BRCA1/2 from sporadic tumors were negative Ki67, N, and M status. In multivariate analysis, the only independent factors differentiating familial non-BRCA1/2 tumors from sporadic ones were low cyclin E expression and negative nodal status. Cyclin E and D1 expression among families with two affected cases was similar to that among sporadic tumors.

Table 8 *Logistic regression analysis (first and final step) of breast cancer features of familial non-BRCA1/2 patients in comparison with breast cancers of sporadic patients.*

Feature	OR (95% CI)	P-value
<i>First step:</i>		
Nodal status neg	1.38 (1.01-1.86)	0.04
M neg	1.42 (0.60-3.73)	0.42
High cyclin E	0.49 (0.32-0.73)	0.001
Low cyclin D1	0.78 (0.51-1.17)	0.22
<i>Final step after stepwise regression:</i>		
High cyclin E	0.56 (0.42-0.75)	<0.0005
Nodal status neg	1.40 (1.04-1.89)	0.03

Table 9 *Cyclin E and D1 expression among different patient groups.*

	BRCA1 tumors (n=53)	BRCA2 tumors (n=58)	Familial non-BRCA1/2 tumors (n=456)	Sporadic tumors (n=439)
Frequency of high cyclin E tumors (n, %)	37 (88.1%)	26 (57.8%)	144 (38.4%)	215 (54.3%)
OR, 95% CI, P-value compared with familial non BRCA1/2 tumors	11.87 (4.56-30.70) <0.00005	2.20 (1.17-4.11) 0.01	- -	1.91 (1.43-2.54) <0.00005
OR, 95% CI, P-value compared with sporadic tumors	6.23 (2.40-16.18) <0.00005	1.15 (0.62-2.15) 0.66	0.52 (0.39-0.70) <0.00005	- -
Frequency of low cyclin D1 tumors (n, %)	37 (84.1%)	33 (68.8%)	167 (45.3%)	197 (48.9%)
OR, 95% CI, P-value compared with familial non BRCA1/2 tumors	6.39 (2.78-14.71) <0.00005	2.66 (1.40-5.07) 0.002	- -	1.16 (0.87-1.54) 0.31
OR, 95% CI, P-value compared with sporadic tumors	5.53 (2.41-12.69) <0.00005	2.30 (1.21-4.37) 0.009	0.86 (0.65-1.15) 0.31	- -

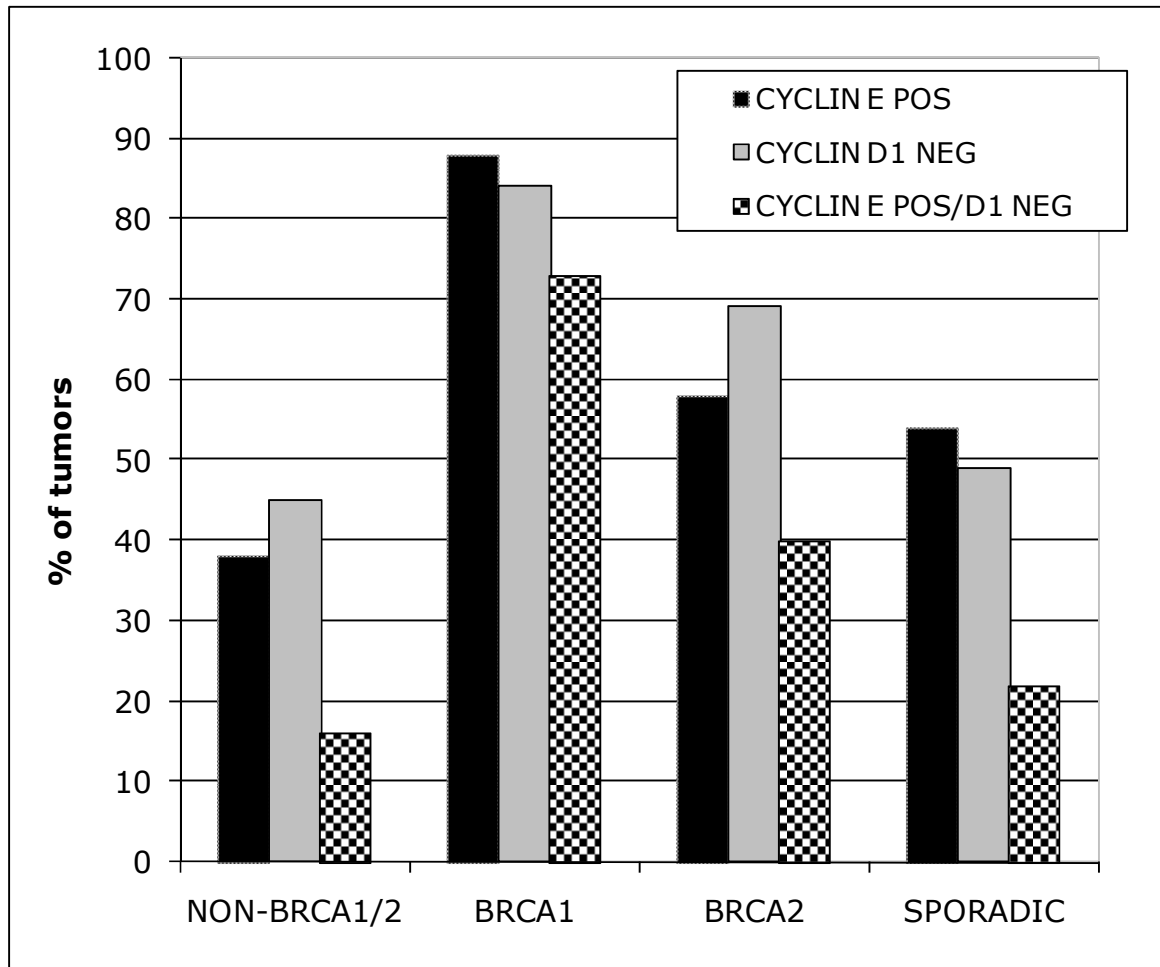


Figure 3 *Percentage of tumors with high cyclin E, low cyclin D1, and high cyclin E/low cyclin D1 expression among familial non-BRCA1/2, BRCA1, BRCA2, and sporadic tumors.*

5.3.4 Associations of cyclin D1 with other tumor characteristics and survival

High cyclin D1 expression correlated with low tumor grade ($P=0.01$), positive nodal status ($P=0.02$), ER ($P<0.0005$) and PR ($P<0.0005$) positivity, and negative p53 immunohistochemistry ($P<0.0005$). Among ER-negative tumors, high cyclin D1 was associated with low grade ($P=0.08$) and low Ki67 expression ($P=0.01$), but among ER-positive tumors, with high grade ($P<0.0005$), high Ki67 ($P<0.0005$), and high cyclin A ($P<0.0005$) expression. Of ER-positive grade 3 tumors, 67% had high cyclin D1 expression, while only 21% of ER-negative grade 3 tumors showed high expression. The correlations between cyclin D1 and grade as well as Ki67 expression were similar among HER2-positive and -negative cases irrespective of ER status. When all tumors were analyzed together, cyclin D1 and E as continuous variables did not correlate ($P=0.99$), but

among ER-positive cases they had a positive ($P<0.0005$) and among ER-negative cases a negative ($P=0.004$) correlation. The frequencies of ductal and lobular histology were not related to cyclin D1 expression, but medullar histology was more common among tumors with low than high cyclin D1 ($P=0.007$). Cyclin D1 expression was not associated with survival among all patients, but showed a trend towards poor MFS among ER-positive patients not given adjuvant chemotherapy (chemotherapy-naïve patients) (RR 1.74, 95% CI 0.93-3.24, $P=0.08$).

5.3.5 Associations of cyclin E with other tumor characteristics and survival

High cyclin E expression correlated with high grade ($P<0.0005$), large tumor size ($P=0.009$), ER ($P<0.0005$) and PR ($P<0.0005$) negativity, HER2 ($P<0.0005$) and p53 ($P<0.0005$) positivity, high Ki67 ($P<0.0005$) and high cyclin A ($P<0.0005$) expression, and younger age at diagnosis ($P<0.0005$). All cyclin E correlations, except the correlation with cyclin D1 expression, as indicated above, were similar among ER-positive and -negative tumors. Ductal and medullar histology were more common among tumors with high than low cyclin E expression ($P<0.0005$ and $P=0.0008$, respectively), but lobular histology was seen more frequently in tumors with low cyclin E ($P<0.0005$). High cyclin E expression correlated with poor MFS in univariate (RR 1.77, 95% CI 1.21-2.61, $P=0.003$) and multivariate (RR 1.76, 95% CI 1.17-2.64, $P=0.006$) analyses and with poor OS in univariate analysis (RR 1.62, 95% CI 1.05-2.52, $P=0.03$), but not in a multivariate model including tumor size, nodal status, grade, ER, PR, and HER2 status (RR 1.01, 95% CI 0.91-2.34, $P=0.41$). Hormone receptor status, chemotherapy, or endocrine treatment did not affect cyclin E associations with survival.

Table 10 *Correlation of cyclin D1 with other tumor characteristics.***A. Among all tumors**

Variable	Correlation coefficient	95% CI	P-value
T	-0.120	-0.081 - 0.035	0.43*
N	0.070	0.013 - 0.127	0.02**
M	0.021	-0.037 - 0.079	0.49**
Grade***	-0.074	-0.131 - -0.017	0.01*
ER	0.373	0.323 - 0.422	<0.00005**
PR	0.297	0.243 - 0.350	<0.00005**
HER2	0.043	-0.015 - 0.101	0.15**
Ki67***	-0.039	-0.096 - 0.018	0.18*
Cyclin A***	0.031	-0.027 - 0.089	0.30*
p53	-0.170	-0.226 - -0.113	<0.00005*
Age at onset	0.011	-0.046 - 0.068	0.69*
Cyclin E***	0.000	-0.057 - 0.057	0.99*

B. Among ER-positive tumors

Variable	Correlation coefficient	95% CI	P-value
T	0.040	-0.027 - 0.107	0.24*
N	0.062	-0.006 - 0.127	0.07**
M	0.036	-0.032 - 0.103	0.30**
Grade***	0.151	0.085 - 0.216	<0.0005*
PR	0.068	0.001 - 0.134	0.05**
HER2	0.073	0.005 - 0.140	0.04**
Ki67***	0.156	0.089 - 0.221	<0.0005*
Cyclin A***	0.261	0.197 - 0.323	<0.0005*
p53	-0.027	-0.095 - 0.041	0.43*
Age at onset	-0.042	-0.109 - 0.025	0.22*
Cyclin E***	0.231	0.166 - 0.294	<0.0005*

C. Among ER-negative tumors

Variable	Correlation coefficient	95% CI	P-value
T	-0.02	-0.142 - 0.103	0.74*
N	0.107	-0.015 - 0.226	0.09**
M	0.026	-0.097 - 0.148	0.68**
Grade***	-0.109	-0.228 - 0.014	0.08*
PR	0.130	0.009 - 0.247	0.04**
HER2	0.192	0.067 - 0.311	0.003**
Ki67***	-0.155	-0.273 - -0.033	0.01*
Cyclin A***	-0.002	-0.125 - 0.121	0.97*
p53	-0.076	-0.198 - 0.048	0.23*
Age at onset	0.036	-0.086 - 0.157	0.56*
Cyclin E***	-0.178	-0.294 - -0.056	0.004*

* Spearman's Rho correlation test

** Mann-Whitney-U test

*** correlation is different among all tumors and among ER-positive and -negative tumors analyzed separately

Table 11 *Correlation of cyclin E with other tumor characteristics.*

A. Among all tumors			
Variable	Correlation coefficient	95% CI	P-value
T	0.077	0.019 - 0.134	0.009*
N	-0.004	-0.062 - 0.054	0.88**
M	-0.011	-0.069 - 0.047	0.71**
Grade	0.391	0.341 - 0.439	<0.0005*
ER	-0.348	-0.399 - -0.295	<0.0005**
PR	-0.288	-0.341 - -0.233	<0.0005**
HER2	0.176	0.119 - 0.232	<0.0005**
Ki67	0.389	0.339 - 0.437	<0.0005*
Cyclin A	0.402	0.352 - 0.449	<0.0005*
p53	0.242	0.186 - 0.296	<0.0005*
Age at onset	-0.146	-0.201 - -0.090	<0.0005*
Cyclin D1***	0.000	-0.057 - 0.057	0.99*
B. Among ER-positive tumors			
Variable	Correlation coefficient	95% CI	P-value
T	0.031	-0.037 - 0.098	0.37*
N	0.026	-0.042 - 0.094	0.45**
M	-0.025	-0.093 - 0.044	0.48**
Grade	0.247	0.183 - 0.309	<0.0005*
PR	-0.059	-0.126 - 0.008	0.09**
HER2	0.141	0.074 - 0.207	<0.0005**
Ki67	0.259	0.195 - 0.321	<0.0005*
Cyclin A	0.261	0.197 - 0.323	<0.0005*
p53	0.137	0.069 - 0.203	<0.0005*
Age at onset	-0.114	-0.180 - -0.047	0.001*
Cyclin D1***	0.231	0.166 - 0.294	<0.0005*
C. Among ER-negative tumors			
Variable	Correlation coefficient	95% CI	P-value
T	0.053	-0.070 - 0.175	0.40*
N	-0.055	-0.176 - 0.068	0.38**
M	0.003	-0.120 - 0.126	0.96**
Grade	0.372	0.261 - 0.473	<0.0005*
PR	-0.191	-0.305 - -0.071	0.002**
HER2	0.082	-0.044 - 0.206	0.200**
Ki67	0.396	0.287 - 0.495	<0.0005*
Cyclin A	0.423	0.317 - 0.519	<0.0005*
p53	0.116	-0.007 - 0.236	0.06*
Age at onset	-0.088	-0.207 - 0.034	0.156*
Cyclin D1***	-0.178	-0.294 - -0.056	0.004*

* Spearman's Rho correlation test

** Mann-Whitney-U test

*** correlation is different among all tumors and among ER-positive and -negative tumors analyzed separately

5.4 Cyclin B1 expression in breast cancer (V)

Cyclin B1 expression was studied in the 1348 invasive breast cancers on TMAs. The staining was successful for 1100 tumors (81.4%). The median cyclin B1 value was 5.0% (range 0-72%).

5.4.1 Cyclin B1 and other tumor characteristics

High cyclin B1 expression was associated with large tumor size ($P<0.0005$), positive nodal status ($P=0.008$), high grade ($P<0.0005$), negative ER ($P<0.0005$) and PR ($P<0.0005$) status, positive p53 ($P<0.0005$), Ki67 ($P<0.0005$) and HER2 ($P<0.0005$) status, high cyclin A ($P<0.0005$) and cyclin E ($P<0.0005$) expression, and young age at disease onset ($P<0.0005$). Ductal and medullar histology were significantly more common among tumors with high than low cyclin B1 expression ($P<0.0005$ and $P=0.0008$, respectively), and lobular histology among tumors with low cyclin B1 ($P<0.0005$).

Tumors with the highest cyclin B1 expression (above 10%) were more frequent among BRCA1 than sporadic (OR 2.8, 95% CI 1.4-5.6, $P=0.003$) or familial BRCA1/2 mutation-negative (OR 4.8, 95% CI 2.3-9.9, $P<0.0005$) patients. Cyclin B1 expression among BRCA2 tumors did not significantly differ from expression among sporadic (OR 1.26, 95% CI 0.55-2.87, $P=0.58$) or familial non-BRCA1/2 tumors (OR 2.16, 95% CI 0.93-5.01, $P=0.07$).

5.4.2 Cyclin B1 expression and survival

High cyclin B1 expression had a significant association with poor MFS (OR 2.48, 95% CI 1.72-3.57, $P<0.0005$) and poor OS (OR 2.58, 95% CI 1.82-3.90, $P<0.0005$) in univariate analysis. The associations with poor MFS and OS were stronger among chemotherapy-naïve patients (OR 3.51, 95% CI 2.05-6.01, $P<0.0005$ and OR 3.74, 95% CI 1.96-7.12, $P<0.0005$, respectively). Among the subgroup of patients that had received adjuvant chemotherapy, the association with poor MFS was weaker (OR 1.58, 95% CI 0.96-2.60, $P=0.07$) and no significant association with OS was found (OR 1.56, 95% CI 0.87-2.80, $P=0.13$). For survival analyses, cyclin B1 was dichotomized at the 7th decile (corresponding to 5.6%) since our earlier study had suggested that the optimal cut-off

value for proliferation markers is around the 7th decile. This corresponds to the proportion of grade 3 tumors in our material.

A multivariate model including the TNM status, tumor grade, ER, PR, Ki67, p53, and HER2 status was constructed to analyze the independent impact of cyclin B1 expression on prognosis. When all patients were analyzed, cyclin B1 had a significant independent association with poor MFS (OR 1.68, 95% CI 1.02-2.74, $P=0.04$) and a trend towards poor OS (OR 1.83, 95% CI 0.99-3.40, $P=0.05$). Among chemotherapy-naïve patients, the associations were stronger also in multivariate analysis. With tumor size and nodal status, cyclin B1 was the only factor independently predicting poor MFS (OR 2.31, 95% CI 1.17-4.59, $P=0.016$) and OS (OR 1.79, 95% CI 1.28-4.14, $P=0.04$).

Survival analyses were also assessed separately among subgroups divided by age at diagnosis or ER status, but the results remained similar.

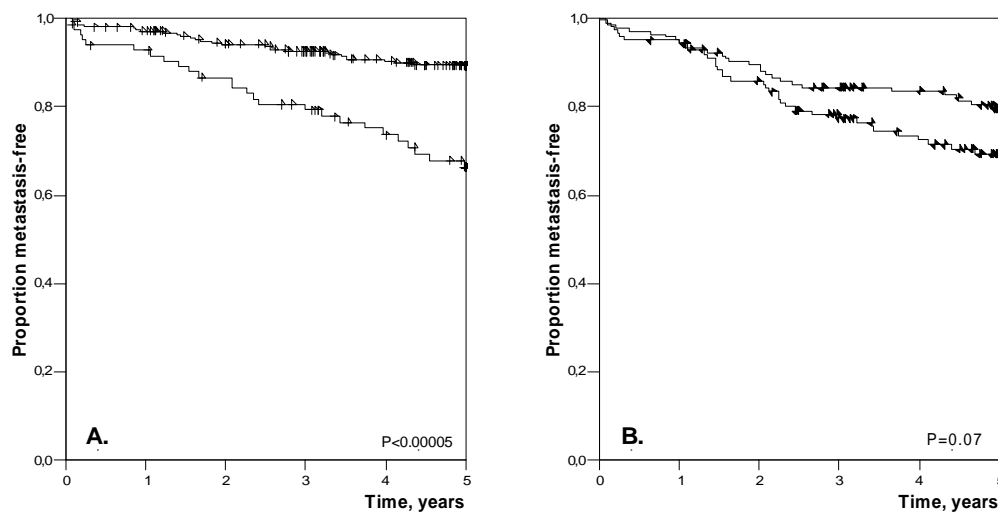


Figure 4 *Kaplan-Meier curves showing metastasis-free survival for cyclin B1 dichotomized at a cut-off of 5.6% among A) chemotherapy-naïve patients and B) patients receiving adjuvant chemotherapy.*

Table 12 *Cyclin B1 expression (dichotomized at a cut-off of 5.6%) and survival in multivariable analysis (Cox regression analysis).*

A. Overall survival			
All patients (n=797)	RR	95% CI	P-value
Nodal status	4.17	2.33-7.49	<0.0005
PR	1.99	1.10-3.61	0.02
HER2	1.91	1.15-3.18	0.01
Tumor size	1.87	1.47-2.37	<0.0005
Grade	1.79	1.14-2.79	0.01
Cyclin B	1.83	0.99-3.40	0.05
P53	1.15	0.91-1.47	0.25
Ki67	1.08	0.62-1.25	0.28
ER	0.89	0.45-1.76	0.74
Chemotherapy-naïve patients (n=473)	RR	95% CI	P-value
Nodal status	3.41	1.55-7.49	0.002
Tumor size	2.87	1.95-4.22	<0.0005
Cyclin B1	1.80	1.28-4.14	0.04
HER2	2.03	0.82-5.02	0.126
Ki67	1.79	0.82-3.79	0.20
PR	1.75	0.73-4.17	0.21
ER	1.70	0.52-5.55	0.38
Grade	1.43	0.78-2.63	0.25
P53	1.15	0.74-1.80	0.54
B. Metastasis-free survival			
All patients (n=797)	RR	95% CI	P-value
Nodal status	2.97	1.87-4.67	<0.0005
Cyclin B1	1.68	1.02-2.74	0.04
Tumor size	1.64	1.33-2.03	<0.0005
Grade	1.63	1.14-2.32	0.008
HER2	1.46	0.92-2.31	0.11
PR	1.39	0.84-2.31	0.20
Ki67	1.26	0.67-1.52	0.26
P53	1.14	0.92-1.41	0.23
ER	0.81	0.45-1.47	0.49
Chemotherapy-naïve patients (n=473)	RR	95% CI	P-value
Nodal status	2.76	1.48-5.13	0.001
Cyclin B1	2.31	1.17-4.59	0.02
Tumor size	1.91	1.35-2.72	<0.0005
Grade	1.44	0.90-2.32	0.13
Ki67	1.39	0.82-1.65	0.35
PR	1.27	0.60-2.67	0.53
ER	1.10	0.36-3.36	0.87
HER2	1.10	0.46-2.63	0.82
P53	1.06	0.72-1.55	0.79

6 DISCUSSION

6.1 Cyclin A expression can be reliably assessed on TMA (I)

The TMA technique allows a large number of tumors to be analyzed simultaneously, thus enabling relatively rapid screening of multiple markers. Cyclin A shows varying expression in different parts of the tumor. When this study was started, no cyclin A analyses on TMA had yet been published. Cyclin A expression in Study I was investigated on TMA and on traditional large sections of 200 breast cancers. The agreement between these two methods was good, as was the reproducibility of the results by two independent readers.

The kappa values describing the agreement of two readers' results were 0.87 for TMA average, 0.83 for TMA maximum, 0.71 for large-section average, and 0.80 for large-section maximum values. Kappa values from 0.61 to 0.80 are considered to reflect good agreement and those from 0.81 to 1.00 very good agreement (Altman 1991). Thus, the reproducibility of cyclin A assessment for the two readers in this study was good or even very good in both large sections and TMAs. Discrepant findings were associated with a low number of counted nuclei, thus a large number of cells counted seemed to improve reproducibility. The agreement between cyclin A assessment on TMAs and large slides using the mean results of the two readers was good for both mean and maximum values. For average values, the agreement was better when the number of cells counted increased. The agreement was moderately weaker for maximum than for average values. This can be explained by the method used in counting cells; on TMA one randomly selected high-power field was counted on each punch, but on large sections we searched one "hot-spot" high-power field and selected the other three randomly. The agreement between TMA and large-section results for cyclin A in this study was similar to that reported for cyclins E and D1 in breast cancer (Han et al. 2003) and for Ki67 in bladder cancer (Nocito et al. 2001).

All histological and prognostic correlations were similar on TMA and large sections. The associations of high cyclin A with ER and PR negativity as well as with high grade are in line with earlier studies (Michalides et al. 2002, Michels et al. 2003). High cyclin A expression was prognostic for distant metastases in both large sections and TMAs, but was

not an independent prognostic factor for overall survival. The results suggest that relative risks for overall and metastasis-free survival can be evaluated on TMA as reliably as on large sections. In conclusion, Study I showed that TMA can reliably be used in cyclin A immunohistochemical assessment of breast cancer.

6.2 Cyclin expression in familial breast cancer (II, V)

6.2.1 Familial BRCA1/2-negative breast cancers show low cyclin E and high D1 expression (II)

In Study II, we investigated the tumor phenotype of familial non-BRCA1/2 breast cancers, focusing on cyclin E and D1 expression, which among this patient group had not been investigated earlier. Moreover, we aimed to find tumor characteristics distinguishing familial mutation-negative cancers from BRCA1/2 positive and sporadic cancers.

Most of the BRCA1 tumors had high cyclin E (88%) and low cyclin D1 (84%) expression. This is in concordance with earlier immunohistochemical studies (Osin et al. 1998, Armes et al. 1999, Vaziri et al. 2001) and cDNA studies showing that high cyclin E expression characterizes the basal-like breast cancer subtype, which is the most frequent cancer type in BRCA1 mutation carriers (Hedenfalk et al. 2001, Sorlie et al. 2003, Foulkes et al. 2004). High cyclin E and low cyclin D1 expression were strong predictors of BRCA1 mutation also when comparing BRCA1 tumors with familial non-BRCA1/2 cases in multivariate analysis. Between BRCA1 and sporadic tumors, however, the only independent differentiating factors were ER, HER2, and age at diagnosis.

Of BRCA2 tumors, 58% had high cyclin E and 69% low cyclin D1 expression. High cyclin E and low cyclin D1 expression were also independent predictors of a BRCA2 mutation in multivariate analysis when compared with familial non-BRCA1/2 tumors, but did not differentiate BRCA2 tumors from sporadic ones. Our data suggest cyclin E and D1 expression can be used as a novel marker for predicting both BRCA1 and BRCA2 mutation among familial breast cancer cases. This conclusion has not been reported earlier. Identifying novel biological characteristics that predict BRCA1 and/or BRCA2

mutation among breast cancer families is of high clinical importance, as patients are usually referred for genetic counseling and testing based on a family history of cancer.

Another important new finding of Study II was that familial non-BRCA1/2 tumors differed significantly from sporadic breast tumors by having significantly lower cyclin E expression. Higher cyclin D1 expression differentiated these tumors from BRCA1- and BRCA2-associated tumors. Cyclin E and D1 were independent factors characterizing familial non-BRCA1/2 tumors in multivariate analysis as well. Similarities in cyclin E and D1 expression that reflect crucial events in cell cycle control and tumorigenesis among BRCA1 and BRCA2 tumors probably also reflect similarities in the functional roles of these two tumor suppressor genes in DNA repair, transcriptional regulation in response to DNA damage, and breast cancer predisposition (Yoshida & Miki 2004). The significantly different expression of cyclin E and D1 among familial non-BRCA1/2 tumors compared with both BRCA-associated and sporadic tumors may indicate that at least part of this group has unique biological characteristics and a genetic background that distinguishes it from both BRCA1 and BRCA2 tumors as well as from sporadic tumors. Whatever genetic change underlies these characteristics, it does not seem to involve the BRCA1 or BRCA2 pathways since results for cyclin E and D1 expression in familial non-BRCA1/2 patients are opposite to those in BRCA-mutation carriers.

6.2.2 BRCA1-related tumors have high cyclin B1 expression (V)

In Study V, we investigated cyclin B1 expression in breast cancer. BRCA1 tumors significantly more often had high cyclin B1 (above 10%) expression than sporadic or familial non-BRCA1/2 tumors. To our knowledge, cyclin B1 expression among breast cancers of BRCA1 carriers has not been studied earlier, but this finding is in line with other characteristics of BRCA1-related cancers, e.g. high proliferation and grade. Cyclin B1 expression did not significantly differentiate cancers of BRCA2 carriers from sporadic or familial non-BRCA1/2 cancers, although high cyclin B1 seems somewhat more common among BRCA2-related than familial non-BRCA1/2 cancers.

6.3 CCND1 G870A and COMT Met108/158Val high enzymatic activity alleles increase breast cancer risk (III)

In Study II, cyclin D1 and E expression was found to characterize breast cancers of familial non-BRCA1/2 patients as compared with mutation-positive and sporadic patients. In Study III, we furthermore wanted to study the contribution of CCND1 gene polymorphism G870A to breast cancer risk independently and in interaction with COMT gene Met108/158Val polymorphism. These polymorphisms were evaluated in two independent populations from Ontario, Canada, and Finland. Both of these polymorphisms occurred frequently in the population controls: the frequency of CCND1 AA genotype was 21.7% and 21.3%, and the frequency of COMT ValVal genotype 22.4% and 20.8% among the Canadian and Finnish samples. These frequencies are in line with earlier data published on these polymorphisms.

The CCND1 high (AA) genotype had a significant association with breast cancer risk in both Canadian and Finnish samples. Three earlier studies with relatively small populations (200-500 cases) had found no significant association (Griew et al. 2003, Krippel et al. 2003, Försti et al. 2004). Thus, our study was the first to show a significant independent impact of the CCND1 polymorphism on breast cancer predisposition in two separate, relatively large case control series. Shortly after our study, a Taiwanese study with 992 breast cancer patients and 960 controls showed that AA and AG genotypes were significantly more common among cases than controls, supporting the finding that the CCND1 high (A) allele contributes to breast cancer predisposition (Yu et al. 2008). CCND1 polymorphism has previously been shown to alter the enzymatic activity of its protein product, and the protein encoded by the CCND1 high (A) allele has been hypothesized to produce a more stable protein than the low (G) allele. Cells with the more stable high enzymatic activity allele may remain under proliferative influence longer, thus giving a rationale for this variant in cancer predisposition.

The COMT high (ValVal) genotype had an independent significant association with breast cancer risk in the Canadian but not in the Finnish series. A meta-analysis carried out on the reported case control studies in Caucasian breast cancer samples and noncancer controls showed a significant association of this COMT genotype with breast cancer risk (Lavigne et al. 1997, Millikan et al. 1998, Thompson et al. 1998, Mitrinen et al. 2001,

Kocabas et al. 2002, Wedren et al. 2003, Ahsan et al. 2004, Sazci et al. 2004, Gaudet et al. 2006, Onay et al. 2006, Akisik et al. 2007). Earlier literature has suggested the COMT high enzymatic activity genotype to have a protective role in the cell by accelerating the conversion of catechol estrogens into their proper methoxyestrogens. The enhanced activity has been postulated to reduce the chance of DNA damage caused by reactive oxygen species created by oxidation of estrogen. A recent study, however, reported noncompetitive negative feedback inhibition of CYP1A1 and CYP1B1 enzymes by methoxyestrogens (Dawling et al. 2003). According to this report, methoxyestrogens generated by COMT inhibit oxidation of the parent estrogen by CYP1A1 and CYP1B1. In addition, although one of the metabolites of COMT, namely 2-methoxyestrogen, is found to protect the tissues from cancer by inhibiting angiogenesis (Zhu & Conney 1998), the same product was also found to cause chromosome breaks and aneuploidy at increased concentrations (Tsutsui et al. 2000), suggesting the importance of balance of concentrations of any metabolites or enzymes in the estrogen metabolism. Thus, this data provides a rationale for the role of high COMT activity in breast cancer carcinogenesis.

The combined effect of CCND1 and COMT genotypes on increased breast cancer risk was investigated, since these genes have complementary functional roles in the estrogen pathway. The results imply a genetic cross-talk between the medium and high enzymatic activity allele combinations of CCND1 and COMT in breast cancer development. The functions of both CCND1 and COMT in the estrogen pathway can explain the biological relevance of this combined effect. The reduced estrogen metabolization by the negative feedback of high COMT activity may result in increased levels of estrogen, which in turn may lead to enhanced expression of CCND1. Because the CCND1 high (A) variant also encodes a more stable form of the protein, the cells containing this combination may be more susceptible to cell cycle progression and proliferation. This suggestion warrants further experimental validation. The findings of our study imply that the individuals inheriting the combinations of high activity COMT and CCND1 alleles have relatively higher breast cancer risk probably due to simultaneously reduced estrogen metabolism and increased cell proliferation. The results also show an example of the potential role of combined effect of low penetrance variants on breast cancer predisposition. Recently, a similar model of several low penetrance variants increasing individual risk has been tendered for prostate cancer (Zheng et al. 2008).

6.4 Cyclin expression and tumor characteristics (IV, V)

6.4.1 Cyclin E expression is elevated in aggressive breast cancer (IV)

In Studies II and III, the roles of cyclin D1 and E in familial breast cancer were evaluated. In Study IV, we further investigated cyclin D1 and E expression and their associations with tumor phenotype and survival. Study IV showed that high cyclin E expression correlates with an aggressive breast cancer phenotype, strengthening the role of high cyclin E in breast cancer with aggressive behavior. High cyclin E expression correlated with high grade, high Ki67 and cyclin A expression, ER and PR negativity, HER2 positivity, large tumor size, and younger age at disease onset and had a significant association with a new entity, the so-called triple negative (ER-, PR-, and HER2-negative) breast cancer. In this study, high cyclin E was also more common among tumors with negative p53 immunohistochemistry, confirming the earlier finding of Lindahl et al. (2004) that tumors with high cyclin E had p53 insertions or deletions, and undetectable p53 expression. A potential explanation for this may be that chromosomal instability caused by cyclin E leads to inactivation of tumor suppressor p53. Medullary and ductal histology more often showed high cyclin E expression. The association with medullar histology has been described earlier, and this may be due to cyclin E's ability to modulate the infiltrative behavior of the tumor (Berglund et al. 2005).

6.4.2 Cyclin D1 correlates with high proliferation in estrogen receptor - positive breast cancer (IV)

The associations of cyclin D1 with histopathological features and with prognosis in breast cancer are not fully understood. Especially the association with proliferation and the mechanisms by which cyclin D1 drives cell cycle progression remain unknown. The most important finding of Study IV was that among ER-positive tumors high cyclin D1 expression showed a strong significant correlation with high tumor grade and high cyclin A and E and Ki67 expression, but among ER-negative tumors, it associated with low grade and low Ki67 and cyclin E expression. Our study was the largest by far to examine these aspects and the first to show these differing associations in a large breast cancer material. In previous studies, cyclin D1 has been associated with low tumor grade (van

Dienst et al. 1997, Han et al. 2003, Hwang et al. 2003, Jirström et al. 2005), although some studies have not supported this (Michalides et al. 1996, Umekita et al. 2002, Reis-Filho et al. 2006). High cyclin D1 expression has also been more common among hormone receptor-positive tumors, and in line with this, high cyclin D1 in our study showed a strong association with positive hormone receptor status. The low expression of cyclin D1 among medullary cancers indicates that cyclin D1 does not have a significant role among basal subtype or BRCA1 cancers.

Experimental data have implied that in ER-positive breast cancer cells cyclin D1 is needed to drive proliferation, while in ER-negative cells proliferation proceeds through other, cyclin D1-independent mechanisms (Loden et al. 2002). Our results support this view; we showed that proliferation in ER-positive tumors is mediated by cyclin D1, but in ER-negative tumors is driven by other, cyclin D1-independent mechanisms. This finding is further supported by cyclin D1 and proliferation marker cyclin A having a high correlation in ER-positive but no correlation in ER-negative cancers. It is also in accordance with an earlier study suggesting that in the ER-negative breast cancer cell cycle regulation does not occur through cyclin D1 and ER (Neuman et al. 1997), but proliferation may be activated through deregulation downstream from the pRB node overexpressing cyclin E. In ER-positive breast cancer cells with a pathologically active cell cycle, the deregulated pathway causing cell cycle activation can be hypothesized to be cyclin D1-pRB, and in these cases cyclin E expression may be a physiological consequence of cell cycle activation. The cyclin D1-pRB pathway warrants further investigation to elucidate this hypothesis. If confirmed, this pathway may even provide a means for developing new targeted biological therapies. Promising results in MCF-7 breast cancer cells imply that pharmacological shutting down of the cyclin D1/CDK4 complex inhibits cell proliferation and could be a useful strategy for limiting tumor growth (Grillo et al. 2006).

In conclusion, the results of Study IV suggest that proliferation is driven by different mechanisms in hormone receptor-positive and -negative breast cancers, and that cyclin D1 has a particularly important role in the hormone-receptor positive breast cancer. These findings reveal new biological data on mechanisms of proliferation and cell cycle control as well as on pathogenesis of breast cancer, with also clinical implications.

6.4.3 Cyclin B1 is associated with aggressive breast cancer phenotype (V)

Cyclin B1 expression in breast cancer was investigated in Study V. The results of Study V show that high cyclin B1 expression is common among breast cancers with aggressive behavior. High cyclin B1 expression was associated with large tumor size, positive nodal status, high grade, high Ki67, cyclin A, and cyclin E expression, and ER and PR negativity. Similar associations have been reported in one previous population (Kühling et al. 2003, Rudolph et al. 2003). One smaller study with 73 tumors (Winters et al. 2001) found no correlation between N, T, ER, and p53 status and cyclin B1 expression, but this discrepancy may be explained by the small sample size. High cyclin B1 expression correlated significantly also with HER2 positivity, and this, to our knowledge, has not been reported earlier and is consistent with an aggressive phenotype. Furthermore, in this study, high cyclin B1 was associated with p53 positivity, which is biologically relevant since p53 controls the cell cycle via cyclin B1 (Innocente et al. 1999). The results of Study IV show that multiple biological factors related to an active cell cycle are intercorrelated.

6.5 Cyclin expression and breast cancer survival (IV, V)

6.5.1 Associations of cyclin E and D1 with survival (IV)

High cyclin E was associated with poor overall survival in univariate but not in multivariate analysis. However, cyclin E was found to be an independent marker of poor metastasis-free survival. Most previous studies have reported an association between high cyclin E and poor outcome, but some studies have been unable to show an independent effect on poor survival. Our study supports the role of high cyclin E in aggressive breast cancer, but failed to confirm an independent prognostic role.

Cyclin D1 expression did not correlate with survival in the whole patient material, but among ER-positive chemotherapy-naïve patients revealed a trend towards poor metastasis-free survival. This is in line with earlier findings since most studies to date have shown that cyclin D1 is not a prognostic factor in breast cancer. Instead, a role in tamoxifen resistance has been suggested. Our material was not designed to evaluate endocrine therapy responsiveness, but a poorer prognosis of tumors with high cyclin D1 among ER-

positive chemotherapy-naïve patients supports the idea that cyclin D1 may have an oncogenic role particularly in hormone receptor-positive breast cancer.

6.5.2 High cyclin B1 predicts poor breast cancer survival (V)

Study V shows that high cyclin B1 expression is a strong independent predictor of poor overall and metastasis-free survival. The association with poor outcome was stronger among chemotherapy-naïve patients. In multivariate analysis, high cyclin B1 predicted shorter overall survival, with RR 1.83 ($P=0.05$), and metastasis-free survival, with RR 1.68 ($P=0.04$), and apart from positive nodal status and large tumor size was the only independent prognostic factor. Among patients receiving adjuvant chemotherapy, cyclin B1 was not an independent predictor of poor survival. Results were similar when ER positive/negative subgroups and patients of different age groups were analyzed separately.

In our material, high cyclin B1 count was a stronger prognostic factor than proliferation markers cyclin A and Ki67, or cyclin E (Table 13). Furthermore, in multivariate analysis, among chemotherapy-naïve patients high cyclin B1 predicted metastasis with a stronger relative risk than tumor grade, PR, or HER2 status, and poor survival with as strong a risk as PR status and grade. The independent prognostic value of cyclin B1 was as strong as or even stronger than the risks with the commonly used biological markers in breast cancer. The independent relative risk for histological grade has been reported to be approximately 1.70-3.20 (Simpson et al. 2000, Elston & Ellis 2002, Volpi et al. 2004), for HER2 2.56 (Joensuu et al. 2003), and for tumor-related proteolytic factors uPA and PAI-1 in a pooled analysis of 18 patient populations 2.58-3.12 (Look et al. 2002). Tumor triple negative status has been associated with poor OS (RR 1.8) and with poor MFS (RR 1.5) (Dent et al. 2007). Gene expression profiles have been suggested to add specificity to prognostic evaluation with traditional and immunohistochemical markers. In a validation study, the most extensively investigated profile, the 70-gene prognosis signature, predicted metastases with RR 2.13 (95% CI 1.19-3.82) and mortality with RR 2.63 (95% CI 1.45-4.79) (Buyse et al. 2006). Thus, high cyclin B1 might be a biological risk predictor as strong as the 70-gene profile, while also being more easily adapted for routine use.

Table 13 *Relative risks for poor metastasis-free and overall survival among chemotherapy naïve-patients (cyclin B1, cyclin A, Ki67, cyclin E, and cyclin D1 dichotomized at the 7th percentile).*

	METASTASIS-FREE SURVIVAL			OVERALL SURVIVAL		
	RR	95% CI	P-value	RR	95% CI	P-value
Cyclin B1	3.51	2.05-6.01	<0.0005	3.74	1.96-7.12	<0.0005
Cyclin A	2.26	1.17-4.86	0.004	2.47	1.17-5.54	0.005
Ki67	1.67	0.97-2.88	0.06	1.90	1.01-3.58	0.05
Cyclin E	1.61	0.94-2.78	0.08	1.43	0.75-2.71	0.27
Cyclin D1	1.55	0.89-2.69	0.13	0.77	0.41-1.45	0.42

Our study is the largest one to date showing an association between high cyclin B1 and poor outcome in breast cancer. A previous smaller study with stage I-II tumors revealed no association (Peters et al. 2004), and one with 109 tumors showed an association between poor survival and only nuclear staining (Suzuki et al. 2007). A study with 73 tumors yielded a significant association (Winters et al. 2001). In the hitherto largest study with 332 tumors with a negative nodal status, cyclin B1 was associated with poor prognosis, but not in a multivariate analysis including Ki67 (Kühling et al. 2003). When 273 tumors treated with surgery and postoperative radiation only were analyzed, cyclin B1 was associated with poor overall survival only in premenopausal patients (Rudolph et al. 2003).

Table 14 *Studies evaluating the association between cyclin B1 and prognosis in breast cancer.*

Study	Patients	Univariate analysis	Multivariate analysis
Winters et al. 2001	n=73	nuclear cyclin B1, OS: RR 1.22, P=0.05 nuclear cyclin B1, RFS: RR 1.23, P=0.02 cytoplasmic cyclin B1, OS: RR 1.36, P=0.02 cytoplasmic cyclin B1, RFS: RR 1.57, P=0.001	nuclear cyclin B1, OS: P=0.02 nuclear cyclin B1, RFS: P=0.005 cytoplasmic cyclin B1, OS: P=0.005 cytoplasmic cyclin B1, RFS: P<0.001
Kühling et al. 2003, Rudolph et al. 2004	n=332 n=273	OS: P=0.022 MFS: P=0.021 CT- and ET-naïve patients: NS CT- and ET-naïve premenopausal patients: OS: P=0.04 MFS NS	NS CT- and ET-naïve patients: NS CT- and ET-naïve premenopausal patients: OS: significant MFS NS
Peters et al. 2004	n=56	NS	NS
Suzuki et al. 2007	n=109	nuclear cyclin B1: significant association with both OS and MFS	nuclear cyclin B1: significant association with both OS and MFS
Aaltonen et al. 2008	n=797	all patients, OS: RR 3.26, P<0.0005 all patients, MFS: RR 2.44, P<0.0005 CT-naïve patients, OS: RR 4.07, P<0.0005 CT-naïve patients, MFS: RR 3.47, P<0.0005	all patients, OS: RR 1.83, P=0.05 all patients, MFS: RR 1.68, P=0.04 CT-naïve patients, OS: RR 1.80, P=0.04 CT-naïve patients, MFS: RR 2.31, P=0.02

Cut-off values around the 7th decile gave the best separation between slowly and rapidly proliferating breast cancers in our previous study (Ahlin et al. 2007). This cut-off value was effective for cyclin B1 as well. Cyclin B1 dichotomized at 5.6%, corresponding to the 7th decile, gave a RR of 2.58 (95% CI 1.82-3.90, P<0.0005) for poor OS and a RR of 2.48 (95% CI 1.72-3.57, P<0.0005) for poor MFS. In our material, this cut-off value identifies a similar proportion of patients for the high-risk group and candidates for chemotherapy as the proportion of grade 3 tumors.

Cyclin B1 expression may not be a pure proliferation marker, but may also reflect other biological properties of the tumor, e.g. genomic instability. This is supported by the fact that the median time to first event among patients eventually developing metastases was similar in patients with high and low cyclin B1 expression. High cyclin A and Ki67 expression, by contrast, was associated with a shorter time to metastases.

In conclusion, Study V shows that cyclin B1 is an independent predictor of poor overall and metastasis-free survival in breast cancer. If verified, cyclin B1 immunohistochemistry might provide a method that could easily be adapted for routine use as a prognostic marker in breast cancer. Furthermore, the lower risk ratios for mortality or metastases among patients receiving adjuvant chemotherapy imply that high cyclin B1 may indicate enhanced sensitivity to chemotherapy.

By investigating critical cell cycle regulator protein cyclins, we revealed new aspects of breast cancer predisposition, pathogenesis, and clinical course. Cyclins E and D1 were shown to play a role in familial breast cancer. Differing cyclin D1 expression suggests different pathways to drive proliferation in estrogen receptor-positive and -negative breast cancers. Cyclin expression seems to aid in prognostic evaluation of breast cancer, and based on our study cyclin B1 was the most specific prognostic marker.

7 CONCLUSIONS

I. Cyclin A expression can be reliably assessed on TMA. The agreement of cyclin A results on TMA and traditional large sections as well as the reproducibility of two readers' results are good. TMA is a method that can easily and reliably be adapted for routine use in large-scale analyses to evaluate cyclin expression and histopathological and prognostic associations in breast cancer.

II. Tumors of familial non-BRCA1/2 patients more frequently have high cyclin D1 and low cyclin E expression than tumors of sporadic or BRCA1- or BRCA2-positive patients. The significantly different expression of cyclin E and D1 among familial non-BRCA1/2 tumors may reflect that at least part of this group has unique biological characteristics and a genetic background that distinguishes it both from BRCA1 and BRCA2 tumors and from sporadic tumors. Cyclin E and D1 expression can function as a novel marker for predicting BRCA1 and BRCA2 mutations among familial breast cancer cases. Identifying novel biological characteristics that distinguish BRCA1 and/or BRCA2 mutation-positive from mutation-negative breast cancer families is of high clinical importance since adding tumor characteristics to models predicting the probability for BRCA mutation can help to make these models more accurate.

III. The CCND1 G870A polymorphism is associated with increased breast cancer risk in two independent populations from Finland and Ontario, Canada. The risk for breast cancer is even greater in the combined genotype of high enzymatic activity genotypes of CCND1 (AA) and estrogen metabolism enzyme COMT (ValVal), suggesting that these alleles act in combination and contribute to breast cancer progression. The biological relevance of this combined effect can be explained by their common relationship with estrogen.

IV. Cyclin E is associated with the aggressive breast cancer phenotype and is an independent predictor of poor metastasis-free survival. High cyclin D1 expression is associated with high tumor grade and high Ki67, cyclin A, and cyclin E expression in ER-positive but with low tumor grade and low Ki67 expression in ER-negative breast cancer. These results imply that cyclin D1 has a controversial role, and proliferation is driven by different mechanisms in ER-positive and -negative breast cancers. The results also emphasize the important role of cyclin D1 in tumorigenesis of ER-positive breast cancer.

In ER-negative cancers, by contrast, cyclin D1 appears to have no role in regulation of proliferation. These findings reveal new biological data on the mechanisms of proliferation and cell cycle control as well as on the pathogenesis of breast cancer, with also clinical implications for prognostic evaluation and possibly even for developing new targeted therapies for breast cancer in the future.

V. High cyclin B1 expression correlates with high tumor grade, large tumor size, positive nodal status, estrogen and progesterone receptor negativity, positive HER2 and p53 status, high proliferation rate, high cyclin E expression, and young age at diagnosis. This shows that cyclin B1 overexpression is common among breast cancers with an aggressive phenotype and that multiple biological factors related to an active cell cycle are intercorrelated.

VI. Cyclin B1 is an independent predictor of poor overall and metastasis-free survival in breast cancer. Apart from tumor size and nodal status, cyclin B1 was the only independent marker of poor outcome among patients not given adjuvant chemotherapy. In our material, cyclin B1 was a more precise prognostic factor than proliferation markers cyclin A or Ki67 and predicted poor outcome better than tumor grade or HER2 or PR status. The relationship of cyclin B1 with breast cancer prognosis warrants further investigation, and if verified, this association suggests that cyclin B1 immunohistochemistry is a method that could easily be adapted for routine use, adding information to prognostic evaluations based on traditional prognostic markers. The lower risk ratios for mortality among patients receiving adjuvant chemotherapy suggest that high cyclin B1 expression may also reveal enhanced sensitivity to chemotherapy.

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